# Individual differences in experimental and clinical pain

## Assessment and genetic susceptibility

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PhD Thesis

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**Abbreviations**

| | |
|---|---|
| 5-HT | Serotonin |
| 5-HTT | Serotonin transporter |
| 5-HTTLPR | A length polymorphism in the promoter region of SLC6A4 |
| ANOVA | Analysis of variance |
| CCDC26 | Putative Coiled-Coil Domain-Containing Protein 26 |
| COMT | Catechol-O-methyltransferase |
| CPM | Conditioned pain modulation |
| DCC | Deleted in Colorectal Cancer |
| DNA | Deoxyribonucleic acid |
| DNIC | Diffuse noxious inhibitory control |
| GSDMC | Gasdermin C |
| GWAS | Genome-wide association study |
| $ICC_{2,1}$ | Intraclass correlation coefficients with a two-way random effect model |
| NRS | Numerical rating scale |
| NS | Specific nociceptive neurons |
| NWR | Nociceptive withdrawal reflex |
| ODI | Oswestry Disability Index |
| OPRM1 | Opioid Receptor Mu 1 |
| PAG | Periaqueductal gray |
| RM ANOVA | Repeated-measures analysis of variance |
| RVM | Rostral ventromedial medulla |
| SDdiff | Standard deviation of the mean difference |
| SLC6A4 | Solute carrier family 6 |
| SOX5 | SRY-Box Transcription Factor 5 |
| SNP | Single nucleotide polymorphisms |
| SRD | Subnucleus reticularis dorsalis |
| VAS | Visual analogue scale |
| VLM | Ventrolateral medulla |
| VNTR | Variable number of tandem repeats |
| WDR | Wide dynamic range neurons |

**List of papers**

I. Lie MU, Petriu E, Matre D, Hansson P, Andersen OK, Zwart JA & Nilsen KB. Psychophysical or spinal reflex measures when assessing conditioned pain modulation? Eur J Pain. 2019. doi:10.1002/ejp.1462

II. Lie MU, Winsvold B, Gjerstad J, Matre D, Pedersen LM, Heuch I, Zwart JA & Nilsen KB. The association between selected genetic variants and individual differences in experimental pain. Scand J Pain. 2020. doi:10.1515/sjpain-2020-0091

III. Lie MU, Pedersen LM, Heuch I, Winsvold B, Gjerstad J, Hasvik E, Nygaard ØP, Grotle M, Matre D, Zwart JA, Nilsen KB. Low back pain with radiculopathy; the role of the genetic variants in the genes SOX5, CCDC26/GSDMC and DCC. Submitted to Journal of Pain.

**Summary**

The experience of pain varies considerably among individuals. Identifying individual differences that may explain the development and persistence of pain, as well as determining how to assess such individual differences holds great promise to improve personalized pain treatment. The present thesis explored individual differences in; 1) pain modulation with different assessments, and 2) experimental and clinical pain in relation to genetic susceptibility.

In paper I we observed individual differences in conditioned pain modulation (CPM) effect between two different CPM protocols, and that individuals had relatively high variations in CPM effect from one session to another session in both protocols. The study raises questions about whether CPM are more related to pain perception rather than nociception on a spinal level, and emphasize that we should be cautions to use the investigated CPM protocols in clinical decision making on an individual level.

In paper II we observed that genetic variation in three genes important for serotonin-, catecholamine- and opioid signaling did not explain individual differences in experimental pain. The study indicates that the selected genetic variants; *SLC6A4* 5-HTTLP/ rs25331 A > G, *COMT* Val158Met or *OPRM1* A118G may not have a large impact on pain sensitivity and pain modulation alone.

In paper III we observed that three genetic variants previously associated with back pain in a genome-wide association study could not explain individual differences in clinical pain outcomes in patients with low back pain (LBP) with persistent radiculopathy. The study indicates that the selected genetic variants *(SOX5* rs34616559, *CCDC26/GSDMC* rs7833174 and *DCC* rs4384683) have limited value as prognostic biomarkers in a clinical setting for subjects admitted to a secondary health care institution for an acute episode of LBP with radiculopathy.

The results from the present thesis carry information for further hypothesis about individual differences in pain modulation with different assessments, and individual differences in experimental and clinical pain in relation to genetic susceptibility. This may in turn contribute to a better understanding of pain and personalized pain treatment.

**Sammendrag**

Opplevelsen av smerte varierer betydelig fra person til person. Å identifisere individuelle forskjeller som kan forklare utviklingen og varigheten av smerter, samt å avgjøre hvordan slike individuelle forskjeller måles, kan bidra til å forbedre persontilpasset smertebehandling. Denne avhandlingen utforsket individuelle forskjeller i; 1) smertemodulering med ulike målemetoder, og 2) eksperimentell og klinisk smerte i forhold til genetisk sårbarhet.

I artikkel I observerte vi individuelle forskjeller i smertemodulering (CPM) mellom to forskjellige CPM-protokoller, og at begge protokollene hadde relativt stor variasjon i CPM fra en dag til en annen. Studien reiser spørsmål om hvorvidt CPM er mer relatert til smertepersepsjon enn nocisepsjon på spinalnivå, og understreker at vi bør være forsiktige med å bruke de undersøkte CPM-protokollene i klinisk beslutningstaking på individnivå.

I artikkel II observerte vi at genetisk variasjon i tre gener viktige for serotonin-, katekolamin- og opioidsignalisering ikke forklarte individuelle forskjeller i eksperimentell smerte. Studien indikerer at de utvalgte genetiske variantene; *SLC6A4* 5-HTTLP/ rs25331 A > G, *COMT* Val158Met eller *OPRM1* A118G mest sannsynlig ikke har stor innvirkning på smerteføl somhet og smertemodulering alene.

I artikkel III observerte vi at tre genetiske varianter tidligere assosiert med ryggsmerter i en genomvid assosiasjonsstudie ikke kunne forklare individuelle forskjeller i kliniske smerteutfall hos pasienter med korsryggsmerter med vedvarende radikulopati. Studien indikerer at de utvalgte genetiske variantene (*SOX5* rs34616559, *CCDC26/GSDMC* rs7833174 og *DCC* rs4384683) har begrenset verdi som prognostiske biomarkører i en klinisk setting for pasienter henvist til sekundærhelsetjenesten for en akutt episode av korsryggsmerter med radikulopati.

Resultatene fra denne avhandlingen inneholder informasjon for videre hypoteser om individuelle forskjeller i smertemodulering med ulike målemetoder, og individuelle forskjeller i eksperimentell og klinisk smerte i forhold til genetisk følsomhet. Dette kan på sikt bidra til en bedre forståelse av smerte og persontilpasset smertebehandling.

## 1. Introduction

The experience of pain varies considerably among individuals. This can be observed in experiments where individuals report a highly controlled and standardized stimulus in the range of 'not painful' to 'very painful', or anything in between (1). Such individual differences are also seen in clinical settings, where pain reports of the same injury, disease or surgical procedure vary substantially between patients (2). It is not unreasonable that we differ in both experimental and clinical pain – after all, we differ in everything else in life – so why is this difference so important?

Pain is one of the leading causes of why we seek health care (3) and represents a large economic burden due to health care utilization and lost productivity (4, 5). Pain also often interferes with daily activities, social life and work life, and thereby reduces the individual's quality of life and health (6, 7). Unfortunately, a large proportion of those who suffer from pain, receive inadequate pain treatment (8). One reason for this may be that researchers and clinicians long have failed to take individual differences into account to personalize the treatment to the individual itself. Identifying individual differences that contribute to development and persistence of pain, as well as determine how to assess such individual differences holds great promise to improve pain treatment and is therefore a high priority (9).

The biopsychosocial model is an excellent model for conceptualizing individual differences in pain and how to achieve personalized pain treatment. According to this model, an individual's experience of pain is composed of biological (e.g. genetic susceptibility, nutrition and physiological processes), psychological (e.g. emotions, experiences and personality) and social (e.g. environment, culture, interpersonal relationships and socioeconomics) factors (figure 1) (10). The complex and dynamic interactions between such factors, results in a unique picture of how pain is provoked, modified and maintained in each individual (8), and an understanding of this unique picture is essential in order to optimize the personalized treatment to the individual.

To date, there are too many missing pieces in the puzzle to realize an optimal, personalized pain treatment. To solve the puzzle, we need to study various biological, psychological, and social factors individually and combined.

Endogenous pain modulation is one biological factor that has received increasing interest over the last decade, particularly the conditioned pain modulation (CPM) model. The clinical use of CPM models depends upon valid and reliable methods. To date, there is a large variation in the CPM methodology. Thus, studies comparing differences in magnitude and reliability of CPM protocols are needed (11, 12).

Genetic susceptibility is another biological factor that may explain individual differences in experimental and clinical pain. Genetic variants can potentially be used as biomarkers to guide pain management.

The focus of the present thesis was individual differences in; 1) pain modulation with different assessments, and 2) experimental and clinical pain in relation to genetic susceptibility. Thus, the present thesis will explore biological factors underlying individual differences in pain; in an attempt to put together another piece of the big puzzle of pain and personalized pain treatment.

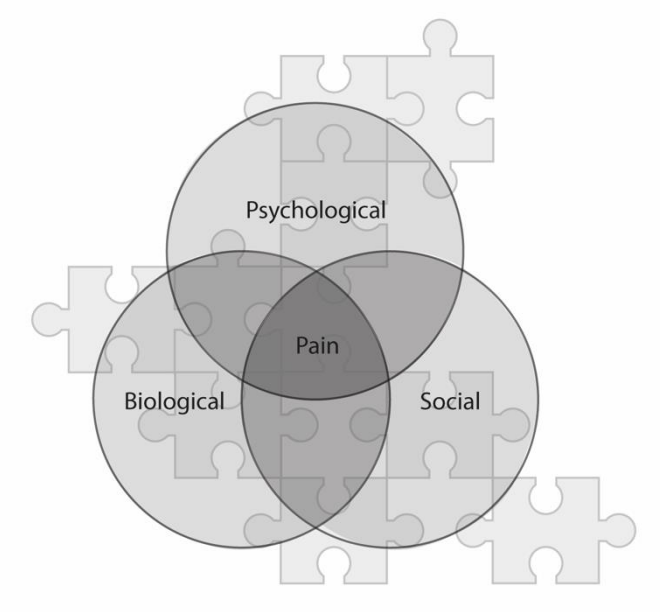


Figure 1. The biopsychosocial model. The experience of pain is influenced by biological, psychological and social factors. The overlapping circles represent the complex and dynamic interactions between the different factors. There are still missing pieces of knowledge for each of the different factors. In the present thesis we focus on biological factors. The illustration is made by the author.

## 1.1 Pain and nociception

### 1.1.1 Definitions and classifications

Pain is a complex phenomenon, which is visible in every aspect of pain, even in the terminology. Many researchers and clinicians have attempted to find a definition for pain that includes every feature of pain, expressed accurately from a scientific basis, simultaneously as being pragmatic for patients and clinicians, but the definitions are never completely satisfactory and often highly debated and moderated (13, 14). The most widely accepted definition of pain is "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (15). According to this definition, pain have an emotional component, which includes several psychological, cognitive and contextual influences, which makes pain a highly complex and subjective experience and not only a physiological response to harmful stimulus (15).

To describe the physiological aspects of pain, the term "nociception" is used. Nociception is the neural process of encoding noxious stimuli and is defined as "pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors." (15). According to this definition, nociception is pain experience based on an observable activity in the nervous system in response to an adequate stimulus (see section 1.1.2 for details). Although pain is a product of nociception and thereby not easily distinguishable, pain and nociception are different phenomena. Substantial stimulation of nociceptors can occur without experiencing pain and pain can occur without stimulation of nociceptors (16).

The various methods to classify pain also reveal the complexity of pain. Pain is often classified according to duration (acute, transitioning, chronic, etc.), localization (back pain, leg pain, etc.), or the possible pathophysiology of the pain (nociceptive, inflammatory, neuropathic, idiopathic, etc.) (17-19). The most common method is to divide pain into acute or chronic. Acute pain is typically nociceptive in nature, e.g. it is provoked by a specific disease or injury and serves a useful biologic purpose – to alert the individual to protect the affected area to prevent further tissue damage or to enhance the healing process (20). Because of the biological nature of acute pain, it is usually also evaluated and treated biomedically (8). Chronic pain is often defined as pain that persists beyond 3 or 6 months (18, 21, 22). Pain exceeding 3 months is not a reliable indicator of tissue damage, as most tissues have shorter healing period than 3 months (23). Chronic pain also often occurs in the absence of any sign

of injury, and the intensity of the pain is often out of proportion to the original injury or tissue damage. The physiological purpose of chronic pain is therefore unclear. Although the definition includes a definite number of months, it is not the duration of pain that distinguishes acute from chronic pain but, more importantly, the inability of the body to restore its physiological functions to normal homoeostatic levels (8, 24).

### 1.1.2 Pain physiology

Pain perception is essential to an organism's survival as it serves as a warning signal that triggers responses intended to prevent or minimize tissue damage. Without the ability to detect pain, the body will be exposed to serious or fatal injuries and infections (25). This alarm function is assured by nociception, which refers to the central and peripheral nervous system processing of potentially harmful stimuli (15). Nociceptors are free nerve endings of the primary afferent neuron, which are capable of transducing and encoding noxious stimuli, e.g. a stimulus that is damaging or threatens damage to normal tissues (15). Nociceptors are located in the skin, muscle, viscera, etc., and respond to thermal, chemical and mechanical stimulations (26). The nociceptors cell body is mostly located in the dorsal root ganglia and have a peripheral and central axon, which connects the nociceptors receptive field in the periphery to the central nervous system (25). When tissue damage occurs in the periphery, chemical substances such as adenosine triphosphate, serotonin (5-HT), bradykinin, histamine, prostaglandins and nerve growth factors binds to different receptors on the nociceptor's membrane, which evokes a receptor potential (25). When the receptor potential exceeds a critical firing level, an action potential is triggered in the first-order neuron. This nociceptive signal is transmitted from the periphery to the dorsal horn in the spinal cord through the axon (27). The conduction velocity of the signal depends on the axon's diameter and thickness of the myelin sheath that surrounds the axon. Nociceptors are classified by fiber types (Aδ fibers and C fibers) based on the axon. Aδ fibers are of medium diameter and have myelinated nerve fibers, which allow the action potential to travel through the axon with a high velocity rate of 15-50 meters/second. C fibers are thin, non-myelinated fibers, where signals travel through the axon with a velocity rate of only 1 meter/second (28). Due to these differences in the conduction velocity, signals from Aδ fibers arrive at the spinal cord before signals from C fibers, which contribute to different pain sensations during stimulation. The Aδ fibers evoke the first sharp or pricking specific pain sensation, while the C fibers evoke the second slowly-developing, long-lasting, burning and diffuse pain sensation (29).

When the nociceptive signals reach the spinal cord, the signal is transmitted to a second-order projection neuron through a synapse by the release of neurotransmitters, predominantly substance P (25). The spinal cord is organized in 10 different laminae, where lamina I-VI forms the dorsal horn (30). Mainly two types of nociceptive neurons are located in the dorsal horn; nociceptive specific neurons and wide dynamic range (WDR) neurons (31). Nociceptive specific neurons are located in lamina I-II and receive signals from Aδ and C fibers. WDR neurons are located in lamina IV-V and are polymodal convergent neurons that are activated by both nociceptive and non-nociceptive stimuli (30, 31). After the transduction of the nociceptive signal to the second-order projection neuron in lamina, the nociceptive signal usually crosses the spinal midline and ascends contralateral in the spinothalamic tract through the brainstem to thalamus (25). In the brainstem, connections are made to areas such as subnucleus reticularis dorsalis (SRD), caudal ventrolateral medulla (VLM), and rostral ventromedial medulla (RVM) in medulla, and to the periaqueductal gray (PAG) in mesencephalon (32, 33). In thalamus, the signal is transmitted through synapses to the third-order neuron to the primary and secondary somatosensory cortex, insula, cingulate gyrus and prefrontal cortex where the signal is interpreted as pain (34).

The nociceptive signals can be modulated at different levels from the periphery to the central nervous system, by both ascending and descending pathways in the central nervous system (33). PAG in mesencephalon is responsible for descending pain inhibition by receiving ascending signals from the dorsal horn in the spinal cord (called bottom-up process) and descending signals from the limbic system (called top-down process), such as amygdala which are responsible for regulating fear and attention (16, 32). PAG projects signals through RVM in medulla to the dorsal horn with ‘on-cells’ or ‘off-cells’, which facilitate or inhibit the incoming nociceptive signals (33, 35). Brainstem regions such as SRD and caudal VLM modulates pain through its reciprocal connections with laminae I, IV-VI in the dorsal horn (35-38). A lesion of SRD decreases nociceptive responses of dorsal horn neurons, while stimulation of SRD results in an increase of nociceptive responses of dorsal horn neurons (36). In contrast, stimulation of the VLM decreases nociceptive responses, while a lesion of VLM produces the opposite effect. This suggests that SRD mainly produces facilitatory pain modulation and VLM mainly inhibitory pain modulation (35).

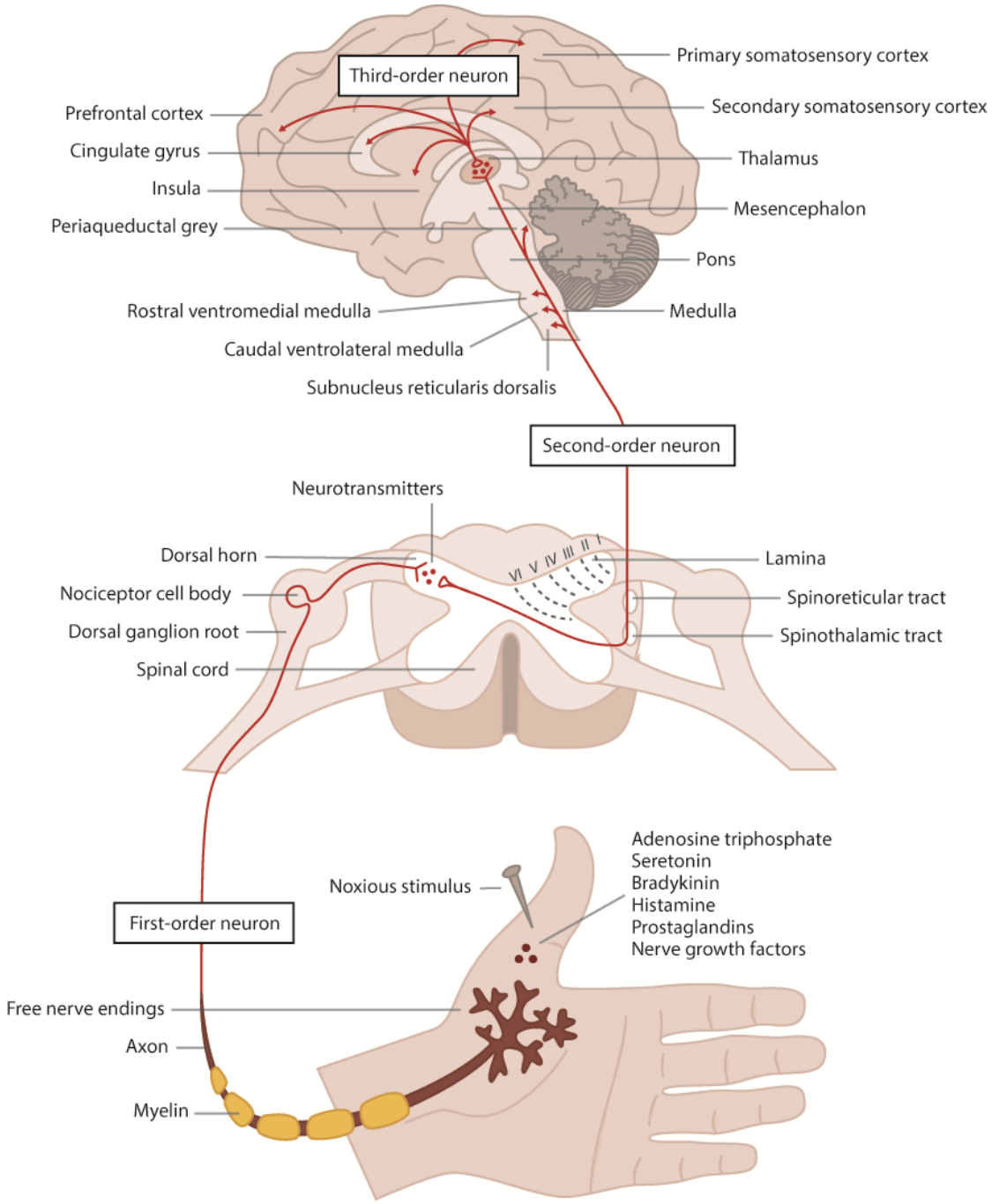


Figure 2. Illustration of the nociceptive processing in the peripheral and central nervous system. A noxious stimulus triggers chemical substances to bind to free nerve endings, which activate a nociceptive signal in the first order neuron. The signal is transmitted to a second-order neuron in the spinal cord and ascend in the spinothalamic or spinoreticular tract through the brainstem to thalamus, where a third-order neuron transmit the signal to different brain structures where the signal is interpreted as pain. The illustration is made by the author.

One of the well-known inhibitory modulation mechanisms is CPM, based on the 'diffuse noxious inhibitory control' (DNIC) concept. The term DNIC was described in the late 1970s after discovering that different stimulations applied to various areas of a rat's body, resulted in

a prominent inhibitory effect in the projection neurons in the spinal dorsal horn. (39, 40). The inhibitory effect was triggered by any type of nociceptive stimuli (i.e. thermal, mechanical, chemical and electrical stimulus), while non-nociceptive stimuli had no inhibitory effect. The DNIC effect in animals have been shown to be mediated by a spino-bulbo-spinal loop. Ascending signals from the spinal cord affects the neural activity in the brainstem, and activate descending signals to the spinal cord. In the spinal cord WDR neurons in lamina V in the spinal dorsal horn are inhibited (41). Studies have shown that DNIC also acts on specific nociceptive specific neurons in lamina I-II (42, 43), but non-nociceptive neurons are not affected by nociceptive stimuli (40). Both Aδ and C fibers have shown to be inhibited. The degree of inhibition is proportional to the intensity and duration of the applied nociceptive stimulus (39). The brainstem regions SRD (44, 45), RVM (41) and PAG (46-48) have been shown to be critical for the DNIC effect.

In humans, DNIC is typically studied by measuring the change in perception of a painful stimulus (test-stimulus) when a second painful stimulus (conditioning stimulus) is inflicted (49). In humans, it is not possible to monitor the neural activity in the same way as in animals. It is thus unknown if the noxious stimulations activate the same neural mechanisms as observed in experimental animal studies. The term conditioned pain modulation was therefore suggested to describe the DNIC-phenomenon in humans (49, 50).

The underlying mechanisms of CPM are assumed to be primarily bottom-up. During assessment of CPM, there is reported activity in SRD, RVM and PAG, but also in higher neural regions such as amygdala, prefrontal cortex, insula and the cingulate cortex (37, 47, 48, 51, 52). Opioids are also suggested to play an important role in CPM (53). When it comes to interrelations between CPM and psychological and cognitive mechanisms, it is suggested that CPM interacts with expectations (54-56), is influenced by pain catastrophizing (57), and acts independently from distraction, but with possible overlaps (58). These results emphasizes that CPM is not a completely separate neural bottom-up mechanism, but also influenced by top-down components.

### 1.1.3 Pain measures

Although pain is a subjective and complex experience, we attempt to quantify it with simple assessments in both experimental and clinical settings in order to understand and evaluate the pain experience and the mechanisms associated with it (59) . When assessing an outcome to

evaluate treatment or to investigate mechanisms, one assumes that the outcome variable is a relatively stable measure. However, pain may fluctuate from time to time (60), and is influenced by many internal and external factors (10), which may decrease the precision of the estimates. There is no gold standard in how to assess pain, and no single assessment method is able to capture the complexity of the experience. Therefore, it is important to identify and use several reliable and valid methods to assess pain to provide effective pain management and ensure high quality research of pain (59).

#### 1.1.3.1 Measures of experimental pain

Pain models are essential for the study of mechanisms and development of treatments (61). Pain models are often developed in animal studies, where the neuronal nociceptive activity can be recorded or behavioral responses can be assessed. However, neuronal recordings or behavior responses do not reflect all the aspects of pain such as psychological and social factors, which makes it challenging to transfer directly into clinical practice (62). Thus, human experimental pain studies acts like a translation bridge between animal experiments and clinical research and practice (63). In experimental pain studies, different stimulation methods are used to activate the pain system in a standardized and reproducible manner. By measuring the following pain response, the effect of the manipulation can be carried out. Human experimental pain studies are limited as it is not possible to determine the exact activated pathways or pain mechanisms (64), but can provide important knowledge in predicting outcome or management of pain (65).

Assessment of experimental pain can be based on psychophysical or neurophysiological methods (66). Psychophysical methods are measurement of the subjective experience of pain, based on standard scales or pain thresholds, while neurophysiological methods are measurement of nociceptive withdrawal reflexes or evoked brain potentials. Psychophysical or neurophysiological methods can be used to measure pain sensitivity, e.g. pain threshold and pain tolerance, or tests that assess the dynamic function of pain modulation, e.g. CPM, offset analgesia, temporal summation and secondary hyperalgesia, which are included in the present thesis. CPM represents reduced pain perception of a painful stimulus (test-stimulus) when a second painful stimulus (conditioning stimulus) is inflicted (49). Offset analgesia is a measure of disproportionate decrease in pain perception after a small decrease in stimulus intensity (67). Temporal summation represents an increase in pain perception despite no change in stimulation intensity (68, 69). Secondary hyperalgesia is present if tissue beyond an

area of tissue damage (primary hyperalgesia) becomes hypersensitive (70, 71). CPM and offset analgesia assesses the pain inhibitory modulation, while temporal summation and secondary hyperalgesia assesses facilitatory pain modulation (72).

There are many methods to assess pain sensitivity and pain modulation. Stimulations are most often applied to the skin, mainly because of the easy access, and are of mechanical, thermal, electrical, chemical and ischemic modality (65, 73, 74). For thermal stimuli, heat stimulation is applied by a thermode, heat foil or laser pulses, while cold stimulation is applied by a cold water bath, a cold gel bag, ice, a wet alcohol sponge, menthol, or a thermode. Rapid skin heating first activates Aδ fiber, which corresponds to the first pain sensation, followed by a C fiber-mediated second pain. Slow heating is assumed to predominantly activate C fibers (65). Cold sensations are assumed to be mediated by Aδ fibers, while cold pain is mediated by C fibres (75). For mechanical stimulation, pinprick or pressure is often used. Pinprick stimulation is believed to activate predominantly Aδ fibers, while pressure activates both Aδ and C fibers (25). Electrical stimulation can be applied with various electrical stimulator devices connected to electrodes applied to the skin. The devices can deliver different waveforms, frequencies, and duration of the stimulus, which selectivity activates different fibers and nervous structures, and therefore evokes different experiences of pain (62, 65). For chemical stimulations, intradermal injection or topical application of capsaicin or mustard oil is typically used. Chemical stimulations is assumed to activate a larger proportion of C fibers than Aδ fibers (65). For ischemic stimulation, a restriction in blood supply and shortage of oxygen is caused by a compressing tourniquet, resulting in activation of predominantly C fibers (76).

#### 1.1.3.1.1 Measurement of conditioned pain modulation

When assessing CPM, both psychophysical and neurophysiological methods are used. Psychophysical methods are most used, but neurophysiological methods may potentially be more reliable since such measures may be less influenced by cognitive processes (77-79).

CPM can be assessed by a parallel- or a sequential paradigm. In the parallel paradigm, test-stimulus is first given alone and then in parallel with the conditioning stimulus, while in the sequential method, the test-stimulus is first given alone and then immediately after the conditioning stimulus. The parallel method has shown to give greater pain reduction compared to a sequential paradigm, but in the parallel method it can be difficult to distinguish

between a clean physiological inhibitory effect due to nociceptive stimulation and the psychological influences due to distraction (53).

Regarding the conditioning stimulus, cold stimulation on an upper extremity is most commonly used (78). Cold water bath as conditioning stimulus have shown to have higher inter- and intra-reliability compared to ischemic pain and pressure pain as conditioning stimulus (80). Heat stimulation is also frequently used as conditioning stimulus (78). The conditioning stimulus is usually a continuous stimulation from 30 seconds to 2 minutes. It is widely accepted that the stimulus needs to be painful to provide an inhibitory effect (53). However, non-noxious conditioning stimuli have also shown an inhibitory effect (81). A possible explanation for this, could be that long-termed non-noxious conditioning stimulus causes temporal summation and thereby becomes painful (81). Furthermore, an increase in conditioning stimulus intensity may increase the magnitude of the CPM effect (81).

The methods used for the test-stimulus varies more than the methods for the conditioning stimulus. Both thermal, ischemic, mechanical and electrical stimuli applied to either upper or lower extremities are widely used (78). Furthermore, different types of tissue are stimulated (muscles, skin, intestines) (49). The duration varies from brief stimulations of few milliseconds, to persistent stimulation with duration up to several seconds or minutes (53). In addition, the stimulation can be either tonic or phasic. Tonic stimulation often consists of a persistent continuous stimulation from 30-120 seconds, while a phasic stimulation is a repetitive stimulation of shorter duration (82). There is currently no consensus regarding the intensity of the test-stimulus. A common approach, independent of the type of stimulus, is to tailor the intensity to an individual pain level – not intolerable or harmful, but painful enough to detect reductions in pain perception during conditioning stimulus. (53).

One of the biggest challenges regarding assessment of CPM is the large variation in the experimental protocols. In addition to all the different stimulation types and stimulation devices, studies vary regarding intensity, duration, stimulation pattern, time interval, etc. The reliability of the different methods ranges from poor to good depending on the methodology of the assessments as well as the statistical analyses (12). The large variation in the methodology limits the generalization of conclusions for application in daily clinical practice (78), and there is a need for establishing gold standards for assessing experimental pain sensitivity and pain modulation.

#### 1.1.3.2 Measures of clinical pain

Numerous questionnaires are available for assessing pain. Generic unidimensional questionnaires measures pain intensity on a scale, such as Visual Analog Scale (VAS) and Numeric Rating Scale (NRS) (83). The most used method is the VAS, where the subjects rate the intensity of the pain on a visual continuous horizontal 0-10 cm (0-100mm) line, with 'no pain' at the left end and 'worst imaginable pain' at the right. NRS is a simpler alternative, where subjects verbally rate intensity of the pain on a scale between 0-10, where 0 represents 'no pain' and 10 represents 'worst imaginable pain' (84). VAS and NRS have shown to correspond and both methods are found to be valid and reliable in most settings (85). However, these scales reduce a complex phenomenon into a single element. Generic multidimensional questionnaires may capture different mechanisms related to the pain experience as they include several aspects of pain, such as pain quality measured by the McGill Pain Questionnaire or disability related to pain measured by the Chronic Pain Grade Scale (83). Disease-specific questionnaires often measures the functional status affected by the condition of interest, such as Oswestry disability Index (ODI) for back pain. It is preferable to use multiple pain assessment parameters in the same experiment. The different measures can then be validated against each other (86).

All self-report measures are vulnerable to response bias, i.e. where subjects deliberately or undeliberately respond inaccurately or falsely to questions. Such bias occurs when respondents misunderstand the question, are inclined to acknowledge or admit information, alter their response in the direction they believe is expected of them to answer or to please the investigators, or exaggerate their response to qualify for support or a desirable treatment (87). Different pain assessment parameters provide different information about the pain experience and may therefore also be prone to different biases. For example, respondent fatigue may be more present in multidimensional or disease-specific questionnaire than in unidimensional pain scales, as time consuming and complex questionnaires with detailed questions may decrease the motivation and ability needed by subjects to accurately answer the questions (87). Recall bias is also a big concern in self-report measures of pain as questions often are retrospective. Several factors have shown to influence the accuracy of recall. For example, present or recent pain may influence reporting of previous pain, and maximum pain may influence reporting of average pain (88). Pain reporting also becomes less accurate as the time period to be recalled increases (89).

## 1.2 Genetics

### 1.2.1 Basic concepts of genetics

The genome is the complete set of genetic instructions for an organism. The genome consists of deoxyribonucleic acid (DNA), which is made up of by two single strands of deoxyribose sugar and phosphate, connected with four types of nitrogenous bases; adenine (A), guanine (G), thymine (T) and cytosine (C) (figure 3) (90). Purine bases (A and G) are complementary paired with pyrimidine bases (T and C) with hydrogen bonds, to form the coiled double helix (91). The DNA is tightly condensed and packed around histones with chromatins into nucleosomes, which together forms the chromosomes (figure 3) (92). Humans have 23 pairs of chromosomes, which are located in the cell nucleus; 22 pairs of numbered chromosomes (1-22), and one pair of sex chromosomes (X or Y chromosome). Each parent contributes one chromosome of each pair to an offspring, which results in a total of 46 chromosomes (93).

A codon is a set of three nucleotides (deoxyribose sugar, phosphate and one nitrogenous base), where the combination of the three nitrogenous bases codes for a specific amino acid or a stop codon (94). A stop codon signals the termination of the translation process of the specific protein. There are about 20 amino acids in the genetic code, and these amino acids form proteins (95). A gene is a sequence of the DNA that provides the cell with instructions for making specific proteins, which then carries out a particular function in our bodies. Only 3 % of the human genome codes for specific proteins (approximately 21.000 genes) (96).

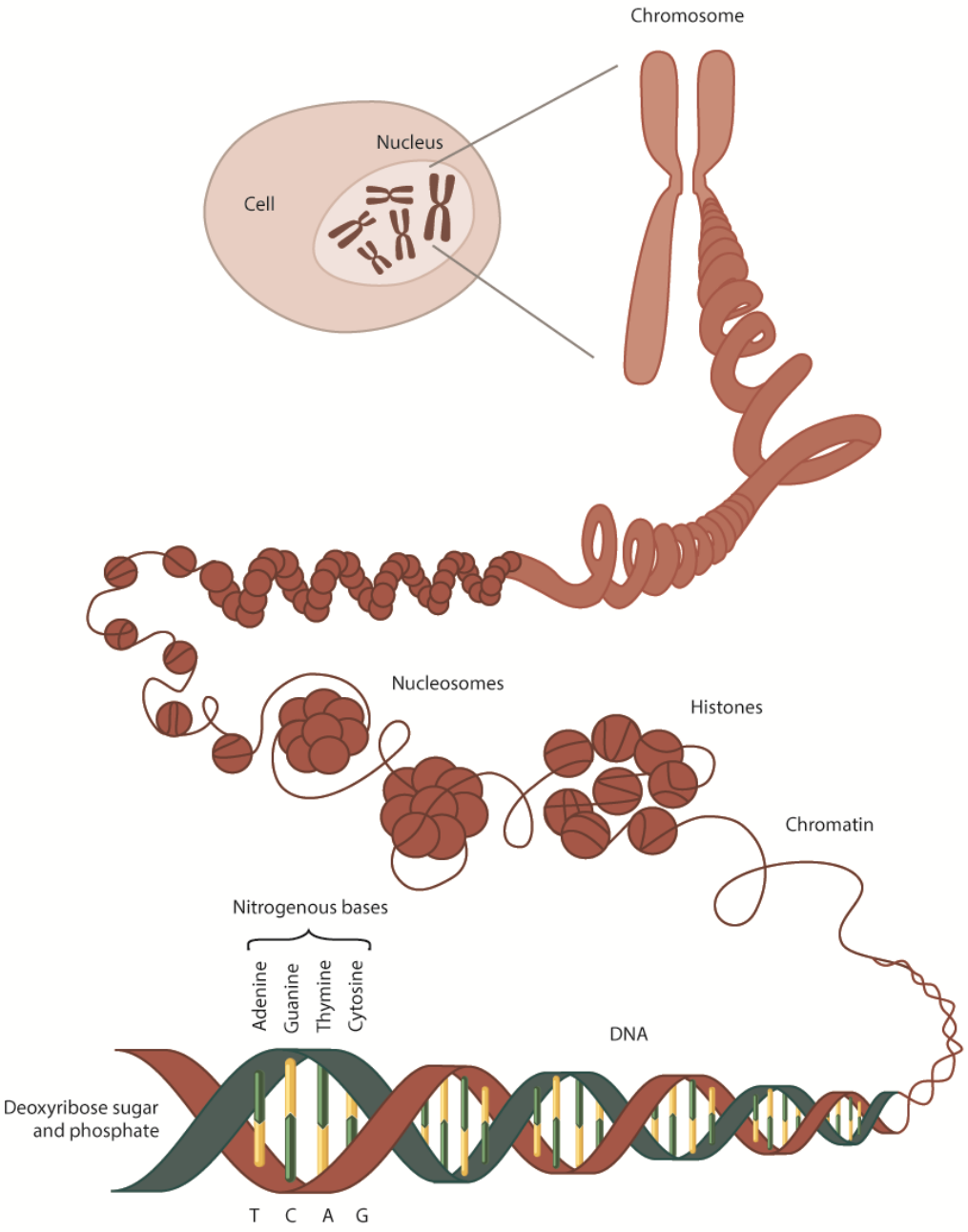


Figure 3. Illustration of the DNA. The DNA is made up by a double helix connected with nitrogenous bases. The strands are packed around histones with chromatins into nucleosomes, which forms the chromosomes located in the cell nucleus. The illustration is made by the author.

### 1.2.2 Genetic variation

More than 99.9% of our DNA sequence is identical to any other human, but a small number of genes vary between people (97). The most common type of genetic variation is single nucleotide polymorphisms (SNP), where a single nucleotide in a specific DNA sequence is changed or missing (figure 4). Most SNPs have no or little effect on protein activity.

However, if the SNP is located in a protein coding region of the DNA and changes the codon to code for a different amino acid (referred to as missense or nonsynonymous mutations) or to a stop-codon (referred to as nonsense mutation), it will influence the shape and function of the protein (93). This may in turn lead to development of a specific trait (98). Another common genetic variation is variable number of tandem repeats (VNTR) (figure 4) (99). VNTR are structural regions of the DNA, where a short nucleotide sequence is repeated a variable number of times in a tandem organization (100, 101). VNTR are classified into microsatellites (repeat sequences shorter than 5 nucleotides) and minisatellites (repeat sequences longer than 5 nucleotides), which are repeated between approximately 5-50 times (93, 102).

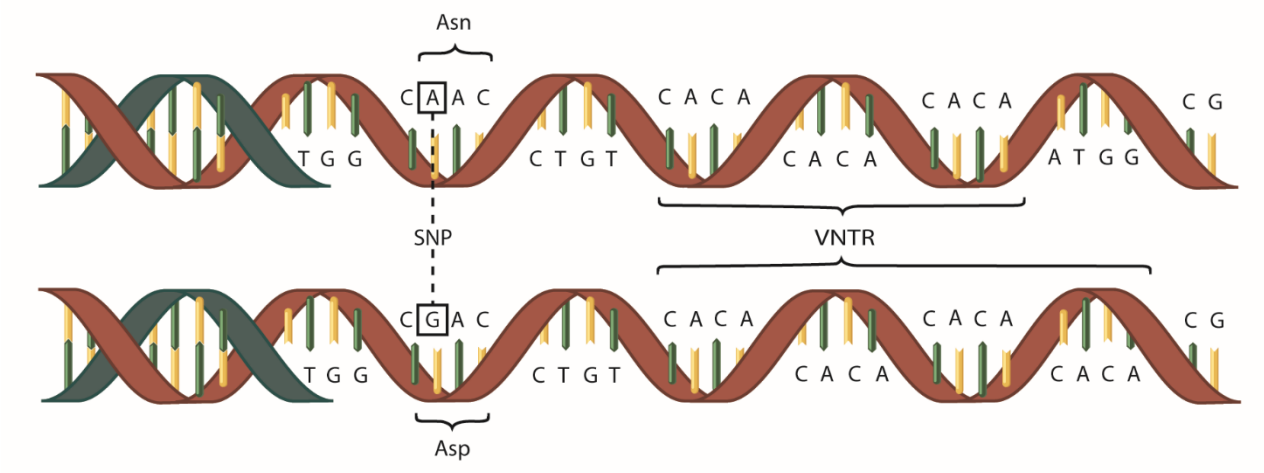


Figure 4. Illustration of a single nucleotide polymorphism (SNP) and variable number of tandem repeats (VNTR) in two different individuals. The SNP is illustrated with rs1799971 where a single nucleotide in the DNA sequence in the OPRM1 gene is changed from A to G, which causes a substitution of the amino acid asparagine (Asn) to the amino acid aspartic (Asp), which in turn influence the function of the protein. VNTR is illustrated with a microsatellite of a repeat sequence of two nucleotides repeated 10 and 14 times resulting in a short and long allele. The illustration is made by the author.

The term genotype refers to the genetic composition of an organism or to the alleles for a particular gene. Because each individual inherits one gene variant from each parent, humans can have two different alleles or two of the same alleles. The major allele is the most common allele for a given SNP, while minor allele is the least common. Risk allele is often used in the context of a disease, and is the allele that is associated with the risk of developing a disease, often the minor allele (92). In regard to VNTR, maternal and paternal copies often results in different length of the two alleles (93). The alleles are classified as short (S) or long (L) depending on the number of repeats.

The term phenotype refers to the observable characteristics of an organism determined by the organism's genotype and environment (103). The way our genes and environment interact to produce a specific phenotype can be a continuous and complicated process. Phenotypes are therefore often dynamic, unstable, and reactive, and therefore difficult to define (103). However, identifying associations between genotypes and phenotypes is critical for increasing our knowledge about disease development and treatment response (104).

### 1.2.3 Genetic studies

There are two main approaches to study associations between genotypes and phenotypes; candidate gene studies and genome-wide association studies (singular: GWAS, plural: GWASs). Candidate gene studies refers to studies that test for an association between a phenotype of interest and one or a small number of polymorphisms, without examining genome wide data (105). The polymorphisms studied are selected based on prior knowledge and hypothesis about gene function or previous associations to the disease of interest. Candidate gene studies are relatively cheap, simple and quick to perform and have been the most widely used approach and the forefront of genetic association studies (105). However, emerging technologies have made it feasible and less expensive to use the GWAS approach, where associations are tested across the entire genome. GWASs have revolutionized the field of complex disease genetics over the past decade, with great success in identifying novel associations between genotypes and phenotypes (106, 107). The challenge with the study design of a GWAS is that the large number of tested SNPs leads to a large proportion of false positive associations. To overcome this limitation, a lower significant threshold is used and a large number of participants is needed (106). Due to the need of large sample sizes in GWASs, the phenotyping and inclusion criteria are often less detailed, leading to a heterogeneous sample. To better translate findings from GWASs into clinical relevance, candidate gene studies with detailed phenotyping and strict inclusion criteria can be used.

Independent of the study approach, the sampling strategy can be crucial for the results of a genetic association study. The case–control study design, where a population with the outcome of interest (cases) is compared to a population without the outcome of interest (controls) (108), has been the most widely applied strategy (109). A case-control study do not require follow-up of patients or waiting for an outcome to occur, and are therefore usually easy, quick and inexpensive to conduct (108). However, case-control studies are often subject for selection bias as the control population often is identified, matched and ascertained after

collection of the cases. A significant difference in allele frequency between cases and controls may appear as an association with the outcome of interest, but be unrelated to the actual influence of the allele under investigation. The association may reflect other differences such as evolutionary or migratory history, gender differences or mating practices (109). This limitation can be improved by using a prospective cohort study design, where collection of individuals is selected before the onset of disease and matched with individuals followed under the same experimental protocol (108). This approach, however, requires significantly more resources in order to obtain a sufficient number of cases.

## 1.3. Physiological and genetic factors associated with pain

### 1.3.1 Different terms of associations

The terms prognostic-, predictive and risk factors are often used to describe indicators for clinical outcomes. However, these terms are often not well defined and are often used interchangeably. Both prognostic and predictive factors are used in the context of foreseeing possible outcomes for individuals who already have developed the disease of interest (110).
A prognostic factor is a measurement that is associated with the progression of a disease. For example, psychological factors such as distress, depressive mood, and somatization have shown to influence the transition from acute to chronic low back pain (LBP) (111).
A predictive factor is a measurement that is associated with response or lack of response to a specific treatment (110). A measurement may be both prognostic and predictive (112). For example LBP patients with higher levels of depression may also respond more favorably to conservative management than surgery (113). A risk factor is a measurement that directly or indirectly increases the probability of a specific outcome. Although the term risk is often used in reference to an individual, whereas the term incidence proportion is used to reference to a group of individuals, the two terms are often used interchangeably (108).

A biomarker is another term used to predict different outcomes, but with a biological focus. It is defined as "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention." (114).
Biomarkers can be predictive-, prognostic- and risk biomarkers depending on what kind of outcome they attempt to measure (115). The study of biomarkers are especially critical for the development of drugs and medical devices (116).

### 1.3.2 Physiological factors and pain

There is substantial evidence that individual differences in experimental pain are related to clinical pain. Increased pain sensitivity have been associated with numerous pain disorders, such as headache, migraine (117, 118), chronic whiplash (119), osteoarthritis (120), fibromyalgia (121) and irritable bowel syndrome (122), and is regarded as one of the characteristics in central sensitization of the nervous system (123). Increased pain sensitivity has also been associated with development of pain after surgery (124-127) and treatment response (128-131). Similar associations have been observed regarding assessments of endogenous pain modulation, such as CPM (132-136), offset analgesia (67, 137-139), temporal summation (140-143) and secondary hyperalgesia (144-146). Assessment of pain sensitivity and pain modulation may therefore be valuable physiological predictive-, prognostic and risk factors in regards to various pain disorders (147). CPM has particularly received a great interest over the last decade. There is, however, large variation in CPM methodology, which limits the generalization of conclusions for application in clinical settings (78). Although CPM is suggested to be a reliable measure, the degree of reliability depends on stimulation parameters (12). A standardized and evidence-based CPM protocol is needed for CPM to be used as a prognostic-, predictive or risk factor and aid personalized treatment (148).

### 1.3.3 Genetic factors and pain

Studies in animals have shown that pain has a moderate to high heritability. When studying heritability in humans, twin studies (in which pain-related traits in monozygotic (identical) are compared to dizygotic (fraternal) twins) are often used to separate genetic and environmental factors. Heritability estimates from twin studies of experimental pain range widely (from 0 % to 60 %), but most studies demonstrate moderate heritability (149). A possible explanation for the variability in heritability may be use of different protocols to assess experimental pain. Cold stimulation has shown relatively consistent estimates across studies (approximately 50 %), while heat stimulations range from 20-53 % (150-152). Twin studies also indicate that clinical pain is heritable. Moderately heritability are observed for pain conditions such as carpal tunnel syndrome, migraine, irritable bowel syndrome, chronic widespread pain, and osteoarthritis (153). However, as for experimental pain, the estimates of heritability of clinical pain also vary between studies. For example, the estimate of heritability for LBP ranges from of 30% to 68% (154, 155). Similarly as in experimental studies, the variability in heritability may be explained by differences in how the outcome measure is assessed. There are numerous

ways to define pain phenotypes (156). For example, although it is widely accepted that chronic pain is defined as pain lasting more than 3 months, the defining question as well as additional definition criteria such as pain intensity and pain frequency, varies substantially between studies (157).

A number of candidate genes relevant for pain have been identified though animal studies and some of these candidate genes have also been further identified in human studies (158). The human pain genetics database is a comprehensive inventory of common genetic variants that are associated with different pain conditions, analgesia and experimental pain. In 2018 it included 294 studies with 434 unique variants across 155 unique genetic loci (159). The number of associations reported in the human pain genetics database demonstrates that experimental and clinical pain are complex traits, which probably are dependent on hundreds of genetic variants in many different genes. However, one of the goals in genetic research is to find genetic variants or genes that may explain a considerable part of the outcome of interest, and thereby function as a biomarker in clinical decision making. The most commonly studied genes in pain genetic research are COMT, OPRM1, SLC6A4, IL1A, IL1B, HLA, TRPV1, GCH1, HTR2A (158, 160-162). These genes are responsible for regulation of different neurotransmitters and receptors involved in pain or temperature signaling (COMT, SLC6A4, TRPV1, GCH1 and HTR2A), cytokines that modulate inflammatory responses (IL1A and IL1B) and molecules important for the immune system (HLA). Several genetic variants from these genes have been associated with assessment of experimental pain as well as clinical pain, especially with musculoskeletal pain conditions (158, 160). Still, there is not enough evidence to use genetic variants from these or any other genes as predictive-, prognostic- or risk biomarkers for pain or pain treatment.

### 1.4 Patient population

LBP is the most common musculoskeletal pain condition globally (163, 164). It is the leading cause of activity limitation and disability (165, 166), which is strongly associated with decreases in the individual's quality of social and working life (7). LBP is also one of the most costly pain conditions due to exceedingly hospitalizations and use of social benefits, e.g. sick leave, disability allowance etc. (6, 167-171). Based on return to function, the majority of LBP patients recover within a few weeks, but approximately two thirds of LBP patients still experience pain 1 year after its onset (172, 173). One of the most common variations of LBP is radiculopathy, in which the patient experiences pain and/or paresthesia in the distribution of

the lumbar spinal nerve due to a nerve root compression (174, 175). Radiculopathy is more persistent, severe, has a less favorable outcome and consumes more health resources than other LBP conditions (175), and may therefore account for a considerable part of the socioeconomic burden of LBP. Intensified research efforts are needed to increase the efficacy of treatment, monitoring and prevention of LBP with persistent radiculopathy. Consequently, this makes LBP with persistent radiculopathy a suitable condition to study in regards to individual differences in clinical pain. The underlying mechanisms for development of LBP with persistent radiculopathy are complex and not fully understood, but genetic susceptibility is assumed to play an important role (176-179). Identifying genetic variants that could serve as prognostic biomarkers would be of great value as in clinical decision making.

## 2. Hypotheses and aims

### 2.1 Overall hypotheses and aim

The overall hypothesis of the present thesis is that individual differences may explain the development and persistence of pain. Identifying such individual differences, as well as how to assess them, holds great promise to improve personalized pain treatment.

One of the underlying assumptions for the present thesis is that it is possible to find reliable and objective methods to measure pain modulation in humans. Therefore, in paper I we hypothesized that assessing conditioned pain modulation (CPM) with spinal reflex produces larger and more reliable CPM effect than psychophysical measures.

Another underlying assumption for the present thesis is that pain perception is genetically determined. Therefore, in paper II we hypothesized that three genetic variants explain some of the individual differences in experimental pain. In paper III we hypothesized that three genetic variants are of clinical relevance for LBP with persistent radiculopathy.

The overall aim of the present thesis was to explore individual differences in; 1) pain modulation with different assessments, and 2) experimental and clinical pain in relation to genetic susceptibility.

### 2.2 Specific aims of the papers

1. Compare the CPM effect and test–retest reliability between a CPM protocol using a thermal test-stimulus and a psychophysical outcome with a CPM protocol with an electrical test-stimulus and a spinal reflex outcome.

2. Investigate associations between each of the selected genetic variants; SLC6A4 5-HTTLPR/rs25531 A>G, COMT rs4680 Val158Met and OPRM1 rs1799971 A118G, and individual differences in experimental pain.

3. Examine if LBP with radiculopathy 12 months after an acute episode of radiculopathy is associated with the selected genetic variants; SOX5 rs34616559, CCDC26/GSDMC rs7833174 and DCC rs4384683.

## 3. Methods

See table 1 for details about design, subjects, recruitment location, recruitment period, inclusion- and exclusion criteria and data collection methods.

Table 1. Overview of the methods of the three papers

| | **Paper I** | **Paper II** | **Paper III** |
|---|---|---|---|
| **Design** | Experimental crossover study | Cross-sectional study | Prospective cohort study |
| **Subjects** | Healthy volunteers | Healthy volunteers and low back pain patients | Low back patients with radiculopathy |
| **Recruitment location** | Advertisement at local hospitals and colleges/universities in Oslo | - Advertisement at local hospitals and colleges/universities in Oslo<br>- Department of Neurology at Oslo University Hospital | - Department of Neurological at Oslo University Hospital<br>- A secondary health care back clinic at the Østfold Hospital Trust<br>- Department of Neurosurgery at St. Olavs hospital, Trondheim |
| **Recruitment period** | September 2016 to December 2016 | January 2013 to June 2018 | January 2013 to June 2018 |
| **Inclusion criteria** | - 18-45 years old<br>- Self-reported to be healthy | - ≥ 18 years old<br>- Self-reported to be healthy and acute low back pain with or without radiating pain rated ≥ 4 on NRS | - ≥ 18 years old<br>- Acute low back pain with radiating pain<br>- Nerve root affection L3-S2 with corresponding clinical manifestations |
| **Exclusion criteria** | - Inability to understand spoken or written Norwegian<br>- Pregnancy<br>- Breastfeeding<br>- Regular use of medication except oral contraceptives<br>- Chronic pain<br>- Somatic or psychiatric disease<br>- Headache for more than two days a month<br>- Hypertension (>140/90)<br>- Acquaintance with the experimenter | - Inability to understand spoken or written Norwegian<br>- Non-Caucasian heritage (mother and father)<br>- Pregnancy<br>- Breastfeeding<br>- Not currently working<br>- Previous or current substance abuse<br>- Regular use of neuroleptics and tricyclic antidepressants<br>- Chronic pain (pain ≥ 4 on 0-10 NRS for ≥ 3 months in the last two years)<br>- Somatic or psychiatric disease<br>- Spinal fracture<br>- Malignancy<br>- Infection<br>- Cauda equina syndrome<br>- Rapidly progressive neurologic deficits | - Inability to understand spoken or written Norwegian<br>- Non-Caucasian heritage (mother and father)<br>- Pregnancy<br>- Breastfeeding<br>- Not currently working<br>- Previous or current substance abuse<br>- Regular use of neuroleptics and tricyclic antidepressants<br>- Chronic pain (pain ≥ 4 on 0-10 NRS for ≥ 3 months in the last two years)<br>- Somatic or psychiatric disease<br>- Spinal fracture<br>- Malignancy<br>- Infection<br>- Cauda equina syndrome<br>- Rapidly progressive neurologic deficits |
| **Data collection** | - Self-reported questionnaires<br>- Experimental pain assessments | - Self-reported questionnaires<br>- Experimental pain assessments<br>- Biological data | - Self-reported questionnaires<br>- Biological data<br>- Data reported by study personnel |

Abbreviations: NRS; numeric rating scale

## 3.1 Data collection

### 3.1.1 Experimental pain

A total of 8 different tests for assessing experimental pain were used in the studies included in the present thesis. See figure 5 for details and illustration of the experimental testing procedures.

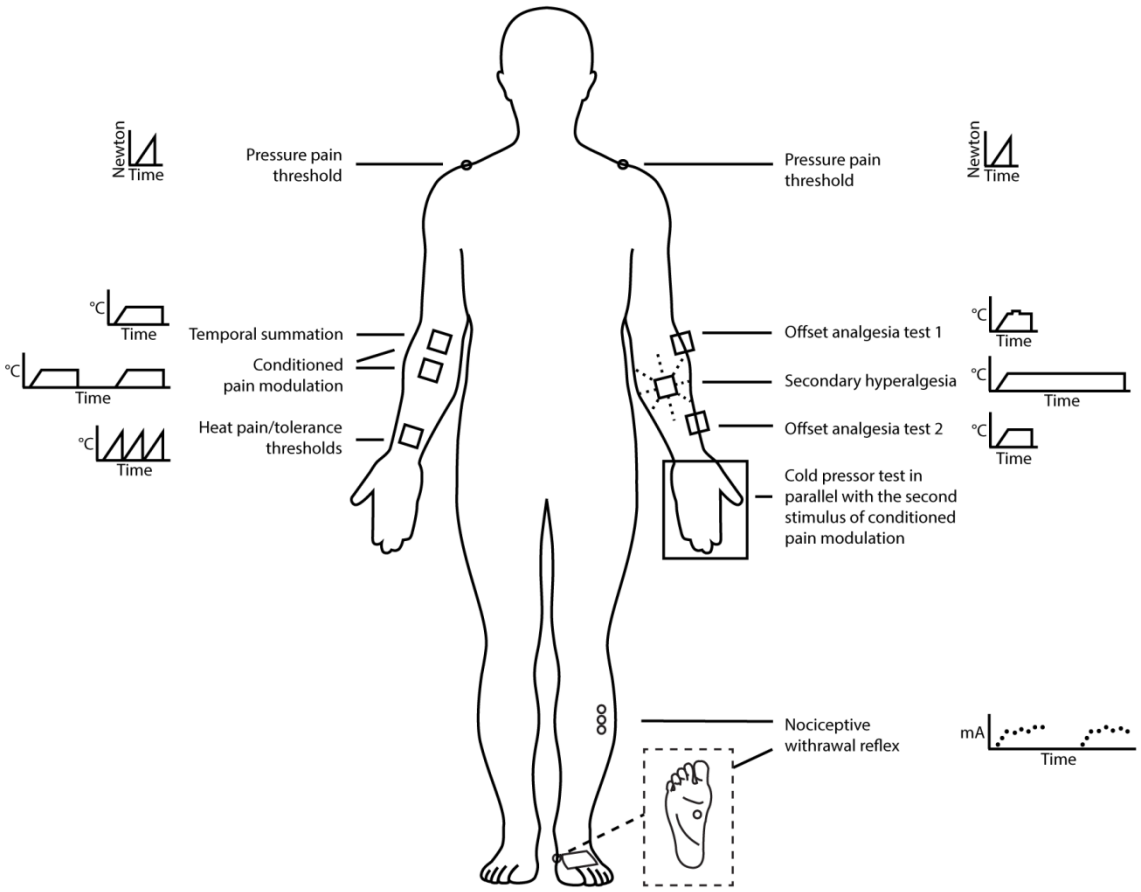


Figure 5. Illustration of the experimental pain testing procedure used in paper I and paper II.
Heat pain threshold and heat pain tolerance threshold was assessed with 3 gradually increasing heat stimulations. To assess pressure pain threshold, increasing pressure was manually applied bilaterally on m. trapezius by a pressure algometer. Temporal summation was defined as an increase in pain ratings >0 on a 10 cm VAS from the start (30-40 seconds) to the end (110-120 seconds) of the stimulation, assessed with a 120 second constant heat stimulation. The stimulation for temporal summation was used as the baseline test-stimulus for assessing CPM in a heat test protocol. After a 5-minute break, an identical test-stimulus in parallel with a 7°C water bath as conditioning stimulus followed. In another CPM protocol using nociceptive withdrawal reflex (NWR) as test-stimulus, an electrical stimulus was induced by an electrode placed on the arch of the foot, and a large electrode placed on the dorsum of the foot. Electromyographic reflex responses were recorded on the ipsilateral tibialis anterior muscle by three electrodes to measure the NWR. Offset analgesia consisted of one trial with 30 s constant heat stimulation and one trial with a 3-temperature paradigm; 5 s constant heat stimulation, followed by another 5 s stimulation increased by 1˚C, before 20 s constant heat stimulation with the initial temperature. Secondary hyperalgesia was assessed with a von Frey filament in 8 directions 45˚ towards the area of primary hyperalgesia induced by a 5 minute heat stimulation of 45˚C. The illustration is made by the author.

In paper I, two different protocols were used to assess CPM. One protocol used a psychophysical approach, where the pain experience of the test stimulus (heat stimulation) was measured on a VAS. The other protocol used a neurophysiological approach, where the test stimulus (electrical stimulations) was measured by the nociceptive withdrawal reflex (NWR). Both protocols used a psychophysical approach to measure the conditioning stimulus (cold stimulation) on an NRS. In paper II, 7 different tests were used to assess experimental pain, which included 3 tests to assess pain sensitivity (heat pain threshold, pressure pain thresholds and heat pain tolerance), 2 tests to assess endogenous pain inhibition (CPM and offset analgesia), and 2 tests to assess endogenous pain facilitation (temporal summation and secondary hyperalgesia). All of the tests in paper II had a psychophysical approach where the experience of pain was measured on a VAS or as a threshold at a specific level of intensity.

### 3.1.2 Clinical pain

In paper III, a clinical outcome measure for LBP with radiculopathy was investigated. To be able to assess the diversity of clinical symptoms in LBP with radiculopathy, 3 different outcome measures were used. Back pain intensity was used as the primary outcome measure, and leg pain intensity and function affected by pain were used as the secondary outcome measures. Back and leg pain intensity was measured in a self-reported questionnaire, where the participants were asked to rate the pain intensity in the past week on a 0-10 NRS. Function affected by pain was measured by the Oswestry Disability Index (ODI), a self-reported questionnaire containing 10 sections regarding intensity of pain, the influence of pain on the ability to take care of oneself, lift, walk, sit, sleep quality, sexual function, social life and travel. Each section is scored on a scale from 0 to 5 with 0 representing no disability and 5 representing severe disability. An ODI score was calculated by dividing the summed score by the total possible score which is then multiplied by 100 (0 = no disability and 100 = maximum disability possible).

### 3.1.3 Genetic variants

The genetic variants investigated in the studies included in the present thesis were selected based on priori hypothesis about the genetic variants and the outcome of interest. The genetic variants investigated in paper II were selected due to their physiological function related to pain. Genetic variants related to serotonin (180), catecholamine (181) and opioid (182) signaling are assumed to be essential for modulating pain perception and are therefore of

particularly interest in regards to individual differences in experimental pain (158). See table 2 for details of the genetic variants investigated in paper II.

Table 2. Details of the genetic variants investigated in paper II selected due to their physiological function related to pain.

| Gene | Protein name | Genetic variant | Chr:position | Allele | Function |
|---|---|---|---|---|---|
| **SLC6A4** | Solute Carrier Family 6 Member 4 | 5-HTTLPR/ rs25531 | 17:30237328 | A>G | An A>G substitution causes reduced expression of the serotonin transporter (5-HTT). A length polymorphism (5-HTTLPR) in the same promoter region results in a short (S) and a long (L) allele, where the S allele also leads to reduced 5-HTT expression. Therefore, 5-HTTLPR and rs25531 are often combined into low ($S_A/S_A$), medium ($SL_G$, $L_A/L_G$, $SL_A$) or high ($L_A/L_A$) 5-HTT-expression types. Expression of 5-HTT plays a central role in the uptake of serotonin in the synaptic cleft. |
| **COMT** | Catechol-O-methyl-transferase | rs4680 | 22:19963748 | A>G | G>A causes a substitution of the amino acid valine (Val) to methionine (Met) at codon 158. This substitution reduces enzyme activity that promote degradation of catecholamines (dopamine, epinephrine, and norepinephrine), which results in higher levels of catecholamines. |
| **OPRM1** | Opioid Receptor Mu 1 | rs1799971 | 6:154039662 | A>G | A>G causes a substitution of the amino acid asparagine to aspartic acid at codon 40. This substitution removes a putative N-linked glycosylation site in the receptor, which is responsible for cellular processes such as receptor folding, sorting, expression and ligand binding. These processes regulate the function of the μ opioid receptor, which impact opioid signaling. |

Abbreviations: Chr; chromosome

The genetic variants investigated in paper III were selected due to previous associations found in a GWAS meta-analysis of chronic back pain (183). This GWAS was based on a heterogeneous sample of back pain disorders, where a considerable proportion of the subjects probably suffer from radiculopathy. It is therefore possible that the genetic variants found in the GWAS are essential for LBP with persistent radiculopathy. See table 3 for details about the genetic variants investigated in paper 3.

Table 3. Details of the genetic variants investigated in paper III selected due to previous associations found in a GWAS meta-analysis of chronic back pain.

| Gene | Protein name | SNP | Chr:position | Allele | Function |
|---|---|---|---|---|---|
| **SOX5** | SRY-Box Transcription Factor 5 | rs34616559 | 12:23794763 | C>T | SOX5 is critical for chondrocyte differentiation during embryonic and notochord development, which may play an important role in the formation of the spine and the intervertebral discs. |
| **CCDC26/ GSDMC** | Putative Coiled-Coil Domain-Containing Protein 26/ Gasdermin C | rs7833174 | 8:129706526 | C>T | CCDC26 is a long non-coding RNA gene, which modulates retinoic acid. Retino acid increases apoptosis. Absence of apoptosis may result in cancer, while excessive apoptosis may result in a cell death of vital cells causing neurodegenerative diseases.<br><br>GSDMC mediates pyroptotic, which defend against intracellular infection by eliminating the compromised cell, thereby removing the pathogen's protective niche, and simultaneously eliciting an inflammatory response. |
| **DCC** | Deleted in Colorectal Cancer | rs4384683 | 18:52852662 | A>G | DCC encodes the netrin-1 receptor, which acts as a tumor suppressor when not bound to netrin-1, and as an axon guidance when bound to netrin-1. Increased DCC expressions can cause sprouting of myelinated afferent fibers in the spinal dorsal horn and thereby cause mechanical allodynia. |

Abbreviations: Chr; chromosome, RNA; ribonucleic acid

## 3.2 Statistical analyses

In all of the studies included in the present thesis, a priori power analysis was undertaken to assure the right sample size for the hypothesis and thereby minimize type II error. See table 4 for details of the calculations.

Table 4. Details from the sample size calculation of the three papers

| | Paper I | Paper II | Paper III |
|---|---|---|---|
| **Number of groups** | 2 | 2 | 3 |
| **Difference between groups** | 1 cm (0-10 VAS) | 10 cm (0-100 VAS) | 1.5 cm (0-10 VAS) |
| **Standard deviation** | 1.5 cm (0-10 VAS) | 20 cm (0-100 VAS) | 1.5 cm (0-10 VAS) |
| **Effect size** | 0.67 | 0.187 | 0.2 |
| **Significance level** | 0.05 | 0.05 | 0.05 |
| **Power** | 0.80 | 0.80 | 0.80 |
| **Sample size** | 20 | 228 | 246 |

All statistical analyses were conducted using SPSS Statistics. The distribution of the data and residuals was always assessed in preliminary analyses by a Shapiro–Wilk test and inspection

of descriptive statistics, histograms, boxplots, and Q-Q plots. See table 5 for detailed description of the statistical analyses performed in the three papers.

Table 5. Details of the main and additional statistical analyses; their objective, method and outcome measures.

| Paper | Analysis | Statistical objective | Statistical analysis | Outcome measures |
|---|---|---|---|---|
| I | Main analyses | Compare the CPM effect between two protocols | RM ANOVA | - CPM effect % (CPM effect/TS × 100) |
| | | Assess the relative reliability of two CPM protocols between two sessions | $ICC_{2,1}$ with absolute agreement definition for single measures | - Pain ratings of TS alone<br>- Pain ratings of TS during CS<br>- Pain ratings of CS<br>- CPM effect |
| | | Assess the absolute reliability of two CPM protocols between two sessions | - Blandt-Altman plots<br>- Mean difference<br>- 95% limits of agreement | - Pain ratings of TS alone<br>- Pain ratings of TS during CS<br>- Pain ratings of CS<br>- CPM effect |
| | | Determine systematic difference in two CPM protocols between two sessions | 1-sample Student's t test. | - Pain ratings of TS alone<br>- Pain ratings of TS during CS<br>- Pain ratings of CS<br>- CPM effect |
| | Additional analyses | Determine whether a CPM effect was present | Paired Sample t-Test | - TS alone vs TS during CS |
| | | Determine the CPM effect for pain- and unpleasantness ratings in the protocol with electrical test-stimulus | RM ANOVA adjusted for changes in thresholds | - CPM effect |
| II | Main analyses | Determine the association between the selected genetic variants and individual differences in experimental pain. | ANOVA or Kruskal-Wallis (non-parametric variables) | - Heat pain threshold<br>- Heat pain tolerance<br>- Pressure pain threshold<br>- CPM<br>- Offset analgesia<br>- Temporal summation<br>- Secondary hyperalgesia |
| | Additional analyses | Compare sample characteristics, individual differences in experimental pain and genotype distributions between healthy volunteers and low back pain patients and patients who had almost or fully recovered from the acute back pain and patients still in a pain state when the experimental tests were performed | - Independent sample Student's t-test (normally-distributed variables)<br>- Mann-Whitney U test (variables with non-normal distribution)<br>- Chi-square or Fisher's exact test (categorical variables) | - Sex<br>- Age<br>- BMI<br>- Education<br>- Smoking status<br>- Hand dominance<br>- Blood pressure<br>- Heat pain threshold<br>- Heat pain tolerance<br>- Pressure pain threshold<br>- CPM<br>- Offset analgesia<br>- Temporal summation<br>- Secondary hyperalgesia<br>- MAF 5HTTLPR/rs25531 *(SLC6A4)*<br>- MAF Val158Met *(COMT)*<br>- MAF A118G *(OPRM1)* |
| | | Determine whether a CPM effect, temporal summation, and offset analgesia was present | Paired sample Student's t-test | - TS alone vs TS during CS<br>- T3-T2 during the three-temperature paradigm vs T3-T2 during the constant stimulation<br>- The start (30–40 s) vs. the end (110–120 s) of the 120 second stimulation |

| Paper | Analysis | Statistical objective | Statistical analysis | - Outcome measures |
|---|---|---|---|---|
| II | Additional analyses | Determine interactions between OPRM1 A118G and sex | Multivariate ANOVA | - Heat pain threshold<br>- Heat pain tolerance<br>- Pressure pain threshold<br>- CPM<br>- Offset analgesia<br>- Temporal summation<br>- Secondary hyperalgesia |
| III | Main analyses | Determine the association between the selected genetic variants and LBP with radiculopathy 12 months after an acute episode of radiculopathy | Kruskal-Wallis H | - Back pain<br>- Leg pain<br>- ODI |
| | Additional analyses | Compare subjects who responded to the follow-up questionnaire at 12 months with subjects who did not respond | - Independent sample Student's t-test (normally-distributed variables)<br>- Mann-Whitney U test (variables with non-normal distribution)<br>- Chi-square (categorical variables) | - Age<br>- Sex<br>- BMI<br>- Education<br>- Smoking status<br>- Baseline back pain<br>- Baseline leg pain<br>- Baseline ODI<br>- Pain duration<br>- Daily medication use<br>- Surgical treatment<br>- MAF *SOX5* rs34616559<br>- MAF *CCDC26/GSDMC* rs7833174<br>- MAF *DCC* rs4384683 |
| | | Determine differences between baseline and 12 months for back pain, leg pain and ODI. | A Wilcoxon Signed Rank test | - Back pain<br>- Leg pain<br>- ODI |

Abbreviations; ANOVA; analysis of variance, BMI; body mass index, CPM; conditioned pain modulation, CS; conditioning stimulus, ICC; intraclass correlation coefficient, ODI; Oswestry disability index, MAF; minor allele frequency, RM ANOVA; Repeated-measures analysis of variance, TS; test-stimulus

### 3.3 Ethical considerations

A written informed consent was obtained prior to participation. The consent included information about the study's background, overall aim, methods, anticipated benefits, potential risks, the discomfort participation may entail, the right to refuse to participate in the study, and the right to withdraw consent to participate at any time without consequences. The study was approved by the Regional committee for medical and health research ethics in Norway (project numbers: paper I; 2010/2927, paper II; 2010/2927 and 2012/1108, paper III; 2013/1060) and conducted in accordance with the Declaration of Helsinki.

To protect the participant's privacy and confidentiality of personal and medical information, all participants received an ID number and were thereby anonymous. The key to identify the ID numbers was stored in a locked archive only accessible to authorized persons. Most medical and recreational DNA testing involves either genotype arrays or full sequencing,

which provide information of hundreds of thousands of genetic variants or the whole genome. Because our DNA is unique one could argue that genetic studies may not be anonymous even when the identification key is destroyed. A study have demonstrated that it is possible to identify DNA contributors even when genetic data from many individuals is pooled in a mixture (184), and law enforcement authorities have identified suspects by distant familial relatives using consumer genomics databases (185). Because of the possibility of identification of anonymous participants in genetic studies it is important to carefully consider how the genetic information is stored, used and published (186). The studies included in the present thesis only sequenced 6 common genetic variants and not the whole genome, and is therefore not subject to identification of individuals without an identification key. The blood samples are stored in a biobank where information is connected via a secure electronic system approved by the Research Support at Oslo University hospital.

The studies included in the present thesis followed the international ethical guidelines for biomedical research involving human subjects (187) in regards to payment for participation. Patients received free follow-up assessments and healthy volunteers received a gift certificate to compensate for the inconvenience, travel costs, time spent and loss of income that may have occurred when participating in the study. The amount of the gift certificate was not advertised in the recruitment process and was not considered large enough to be an incitement for participating in the study by itself. The free follow-up assessments delivered were not crucial for the treatment of pain and would therefore not convince patients to participate beyond consent.

None of the painful stimuli carried out in the experiment in paper I and paper II could cause permanent damages to the participants. The participants were informed that the painful stimuli were not harmful and that they could discontinue the stimulation at any time.

# 4. Results

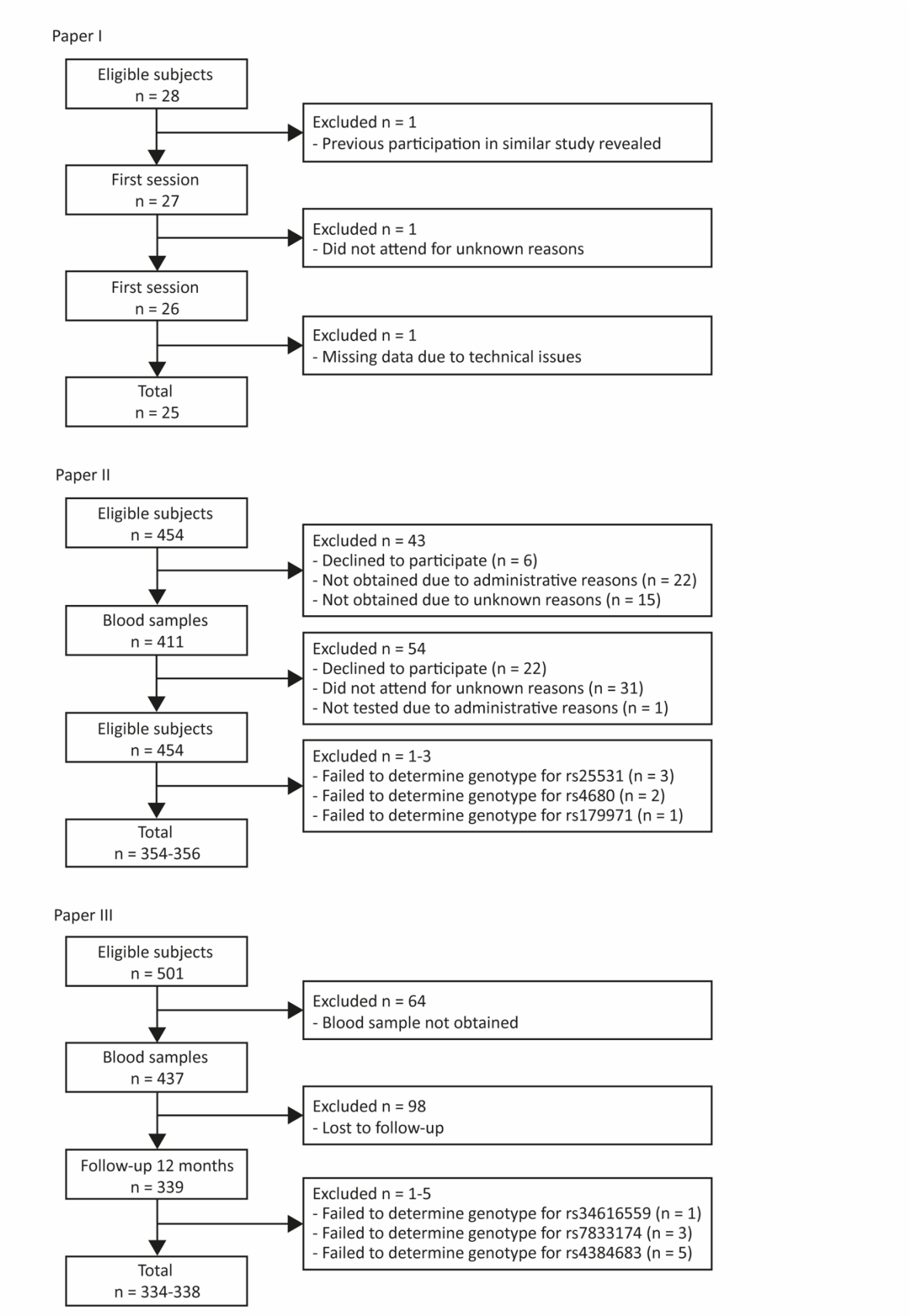


Figure 6. Study flowchart of the three papers.

### 4.1 Paper I

Paper I examined individual differences in CPM effect between two different CPM protocols as well as individual differences in CPM effect between two sessions. The CPM effect and test–retest reliability of a CPM protocol using a thermal test-stimulus and a psychophysical outcome was compared with a CPM protocol with an electrical test-stimulus and a spinal reflex outcome. The study showed a significantly larger CPM effect using a protocol with a psychophysical outcome from using a thermal test-stimulus compared to a spinal reflex outcome using an electrical test-stimulus, where the protocol with a spinal reflex outcome failed to detect a CPM effect. The large difference in CPM effect between the two protocols suggests that the CPM effect relates to pain perception rather than nociception on the spinal level. Fair relative reliability was observed for the CPM effect in both protocols. The absolute reliability indices in both protocols displayed good agreement in the mean CPM effect between the two sessions. However, high intra-individual variability was observed for both protocols. Due to poor absolute intra-rater reliability, we recommend caution and further research before using any of the investigated CPM protocols in clinical decision making on an individual level.

### 4.2 Paper II

Paper II examined whether individual differences in experimental pain were dependent of genetic variation in three genes important for serotonin, catecholamine and opioid signaling. Associations between each of the selected genetic variants; SLC6A4 5-HTTLPR/rs25531 A>G, COMT rs4680 Val158Met and OPRM1 rs1799971 A118G, and individual differences in experimental pain were investigated. The study showed no association between the selected genetic variants, SLC6A4 5-HTTLP/ rs25331 A>G, COMT Val158Met or OPRM1 A118G and individual differences in pressure pain threshold, heat pain threshold, heat pain tolerance, CPM, offset analgesia, temporal summation or secondary hyperalgesia. Thus, the selected pain-associated genetic variants were not associated with individual differences in experimental pain. The finding is an important contribution to the literature, which often consists of studies with lower sample size and one or few experimental pain assessments.

### 4.3 Paper III

Paper III examined if three genetic variants previously associated with back pain could explain individual differences in clinical pain outcomes in patients with LBP with

radiculopathy. Associations between LBP with radiculopathy 12 months after an acute episode of radiculopathy and the selected genetic variants; SOX5 rs34616559, CCDC26/GSDMC rs7833174 and DCC rs4384683 were investigated. A GWAS meta-analysis suggested that the genetic variants *SOX5* rs34616559, *CCDC26/GSDMC* rs7833174 and *DCC* rs4384683 are associated with chronic back pain (183). Since a considerable proportion of the sample used in this GWAS may suffer from radiculopathy, we hypothesized that these genetic variants also would have an impact on subjects with LBP with radiculopathy. However, we found no associations between LBP with radiculopathy 12 months after an acute episode of LBP with radiculopathy and the selected genetic variants. Thus, the study indicates that the genetic variants reported in the GWAS meta-analysis of chronic back pain (*SOX5* rs34616559, *CCDC26/GSDMC* rs7833174 and *DCC* rs4384683) are not of prognostic value in a clinical setting for subjects admitted to a secondary health care institution for an acute episode of LBP with radiculopathy.

# 5. Discussion

## 5.1 Main findings

In the present thesis, we have explored individual differences in; 1) pain modulation with different assessments, and 2) experimental and clinical pain in relation to genetic susceptibility. In paper I we observed individual differences in CPM effect between two different CPM protocols, and that individuals had relatively high variations in CPM effect from one session to another session in both protocols. In paper II we observed that genetic variation in three genes important for serotonin, catecholamine and opioid signaling did not explain individual differences in experimental pain. In paper III we observed that three genetic variants previously associated with back pain could not explain individual differences in clinical pain outcomes in patients with LBP with radiculopathy.

### 5.1.1 Differences in assessment of CPM

In paper I we examined individual differences in CPM effect between two different CPM protocols. We found a significantly larger CPM effect using a protocol with a psychophysical outcome from using a thermal test-stimulus compared to a spinal reflex outcome using an electrical test-stimulus. The large difference in CPM effect between the two protocols is somewhat consistent with previous studies, where studies using 120 seconds heat test-stimulus report a CPM effect between -29 and -47% (188-192), while studies using electrical test-stimulus giving rise to a NWR, report a CPM effect between -11.5 and -40% (193-196). The difference in CPM effect between the two protocols may be attributable to the different physiological effects the two test-stimuli offer. For instance, the two test-stimuli activate different peripheral nerve fibers. In the protocol using thermal test-stimulus, pain sensation produced by Aδ fibers is only present in the first 1-2 seconds of the 120 second heat stimulation, while the following pain sensation is predominately mediated by C fibers (65). In the protocol using electrical test-stimulus, Aδ fibers activate the NWR (77). A CPM effect can be achieved for pain sensations mediated by both Aδ- and C fibers, but a significantly stronger inhibition have been seen in regards to second pain sensation compared to first pain sensation (197, 198), which could explain the larger CPM effect in the protocol using thermal test-stimulus compared to the protocol using electrical test-stimulus. The different duration of the test-stimuli could also activate the central nervous system in different manners (199). Human brain mapping studies have shown that the brain reward system modulated by dopaminergic mechanisms are activated when subjects are exposed to pain and pain relief interchangeably (200). This should have however contributed to larger CPM effects for the

protocol using repeated electrical stimulations, which is inconsistent with the results. The most important physiological difference between the two protocols may be the different impact of cognitive processes between the two test-stimuli. The NWR is commonly considered a proxy for nociception less influenced by cognitive processes than psychophysical measures (77, 201). Although CPM is considered to involve a spinal-supraspinal-spinal feedback loop, CPM have also shown to be highly influenced by supraspinal processes (202). Larger CPM effect using a protocol with a psychophysical outcome compared to a spinal reflex outcome suggests that CPM are more dependent on cognitive/evaluative aspects of pain perception than on nociception, which emphasize the importance of supraspinal processes in the mechanisms of CPM. The difference in CPM effect between the two protocols may also be attributable to the different methodological aspects of the two test-stimuli, such as pain intensity and stimulation sites. In regards to the pain intensity, the NWR threshold has been reported to be correlated with the subjective pain threshold (77), while the thermal test-stimulus was aimed to reflect a pain intensity of 6/10 on a VAS. The CPM effect by the electrical test-stimulus is thereby more prone to a floor effect compared to the CPM effect by the thermal test-stimulus. Individuals are also typically more familiar with noxious thermal stimuli than noxious electrical stimuli. Hence, although the subjects were familiarized with both protocols in a pre-test, the CPM effect during the electrical test-stimulus may also be more prone to modulation from other types of descending control systems, such as e.g. emotions (203), attention/distraction (204), fear or pain catastrophizing (82). In regards to stimulation site, the two protocols may activate different pain modulatory pathways. Two upper limbs are used in the protocol with the thermal test-stimulus, which may possibly reflect a segmental spinal inhibitory effect, while a combination of a lower limb and an upper limb is used in the protocol with electric test-stimulus, which may be more influenced by an ascending-descending modulatory activity. However, differences in CPM effect have not been found when using the same test-stimulus at different locations (205).

In paper I we also examined individual differences in CPM effect between two sessions. We found fair relative reliability for the CPM effect in both protocols. In both protocols the absolute reliability indices displayed good agreement in the mean CPM effect between the two sessions, but high intra-individual variability was observed. In the literature, relative reliability ranges between 0.21and 0.62 for protocols using thermal test-stimulus (206-210), and between 0.26 and 0.61 for protocols using electrical test-stimulus (193, 194). Few studies

have assessed the absolute reliability of the CPM effect (12), and use of different outcome measures challenge the interpretation and comparison across studies, as the level of reliability solely depends on what is acceptable for practical use for the specific outcome measure (211). As both clinical and experimental pain is known to fluctuate (60), and is influenced by psychological and contextual factors that also fluctuate (10), it is natural that CPM is not completely stable over time. The question is how large variation can we accept if CPM is to be used as a risk-, predicting or prognostic factor at an individual level in a clinical setting? According to the calculated absolute reliability in paper I, we can expect a healthy individual that have a CPM effect of -2.2 cm in one session to have a CPM effect in the range of -5.4 cm in or 1.4 cm the next session. The large range observed in paper I may be problematic if we want to test for changes over time or if we use specific cut off-values to guide decisions regarding treatment.

### 5.1.2 Differences in experimental pain

In paper II we examined whether individual differences in experimental pain were dependent of genetic variation in three genes important for serotonin, catecholamine and opioid signaling. We found no association between the selected genetic variants, *SLC6A4* 5-HTTLP/ rs25331 A > G, *COMT* Val158Met or *OPRM1* A118G and individual differences in pressure pain threshold, heat pain threshold, heat pain tolerance, CPM, offset analgesia, temporal summation or secondary hyperalgesia. The literature shows conflicting results regarding associations between experimental pain and *SLC6A4* 5-HTTLPR/rs25531 A > G (212-219), *COMT* Val158Met (220-226), and *OPRM1* A118G (180, 227-231). The inconsistencies may be due to different sample selection and sample sizes, different choice of experimental tests or different tests protocols. Assessments of experimental pain are time-consuming, and as a result, there are few studies with large sample sizes. Paper II is to our knowledge, one of the largest candidate gene study investigating associations between the selected genetic variants and individual differences in both experimental pain sensitivity and pain modulation, and one of the first studies to explore association between the selected genetic variants and offset analgesia and secondary hyperalgesia. One GWAS of an at-home assessment of cold pressure test in 6,853 individuals have been conducted, but the analyses were underpowered and did not identify any significant genome-wide associations (232). Another genome-wide study examined the influence of genetic variation on pain sensitivity in 2,500 human volunteers by exome sequencing in a subset of a twin cohort (233). No genetic variants of large effect were identified due to a stringent multiple test correction, but the *GZMM* gene was classified as

having “very high” evidence of association to thermal nociception. The *GZMM* gene is however, not implicated in pain, but in apoptosis and initiation of inflammation processes. Using pathway analysis, there was significant enrichment for variants in genes of the angiotensin pathway. Angiotensin is a peptide hormone involved in the control of blood pressure, which is modulated by descending brainstem pathways and have been implicated in different pain processes. So far, neither GWASs nor candidate gene studies provide enough evidence to explain individual differences in experimental pain by specific genetic variants. Although GWASs are of great value for determining associations between specific outcomes and genetic variants, it may not be the most appropriate design when investigating individual differences in experimental pain. When examining thousands of subjects the quality of the assessments may decrease due to less time for instructions and familiarization, multiple technicians and technician fatigue (156).

### 5.1.3 Differences in clinical pain

In Paper III we examined if three genetic variants previously associated with back pain could explain individual differences in clinical pain outcomes in patients with LBP with radiculopathy. We found no associations between LBP with radiculopathy 12 months after an acute episode of LBP with radiculopathy and the selected genetic variants; *SOX5* rs34616559, *CCDC26/GSDMC* rs7833174 and *DCC* rs4384683. The findings indicates that the genetic variants reported in the GWAS meta-analysis of chronic back pain have limited value as prognostic biomarkers in a clinical setting for subjects admitted to a secondary health care institution for an acute episode of LBP with radiculopathy. Previous candidate gene studies and GWASs report conflicting results about genetic variants important for LBP with persistent radiculopathy (176-179, 234, 235). Of the genetic variants in SOX5, *CCDC26*/*GSDMC* and *DCC*, it is only the association with genetic variants in the *CCDC26*/*GSDMC* gene that seems to be replicated across studies for LBP with radiculopathy (177, 234, 235). The conflicting results may be influenced by small sample sizes and different phenotype definition.

In the last decade, GWASs have had a great success in identifying novel associations and has added valuable information about underlying mechanisms of the outcome of interest (106). Despite this success, few findings from GWASs are clinical applicable. Although traditional candidate gene studies may seem obsolete in the presence of comprehensive and well-powered GWASs, candidate gene studies have the beneficial to evaluate strong GWAS

candidates for clinical utility in cohorts with detailed phenotyping and strict inclusion criteria. The GWAS meta-analysis of chronic back pain that formed the basis of the selected variants studied in paper III, consisted of over 158,000 individuals, including over 29,000 cases of chronic back pain (183). It is unknown what kind of back pain conditions that are included in the sample. The genetic variants found in this GWAS are thereby not specific for any back pain condition, which reduces the clinical relevance of the finding. The lifetime incidence of LBP with radiculopathy ranges between 10 % and 40 % (236), and we can therefore assume that a considerable proportion of the sample in the GWAS meta-analysis of chronic back suffer from persistent radiculopathy. Since we did not succeed to replicate the significant associations with the same genetic variants in our sample of LBP with persistent radiculopathy, it is possible that these genetic variants are specific for other LBP conditions than LBP with radiculopathy. The negative results may also be a reflection of genetic variants that only explain a small fraction of the genetic contribution to LBP with radiculopathy, and thereby have too small effect size to detect significant associations with the sample size in paper III.

## 5.2 Methodological considerations

### 5.2.1 Design

Paper I was an experimental cross-over study. An experimental study is considered beneficial when investigating the effect of an exposure and a cross-over design is commonly used when investigating several exposures (237). In a cross-over study, each subject undergoes all the exposures, separated with a washout period. As each subject acts as its own control, the variability in the outcome from outside confounders is reduced. Less variation increases the power to detect the effect of the exposure, which allows the statistical inference to be made with fewer subjects compared to independent designs (237). In a cross-over study the order of exposure is counterbalanced to avoid systematic differences between exposures due to practice or boredom effects, which may occur when subjects are exposed to repeated measures (237). In paper I, this was assured by a computerized block-randomization for the order of protocol prior to the experiments. Test side was also counter-balanced as hand dominancy is considered to influence the experimental pain experience (238). To assess systematic differences between the two sessions, bias was calculated and analyzed. The bias was close to zero in both protocols, suggesting no practice or boredom effects between sessions.

Paper II was an experimental cross-sectional study. A cross-sectional study is less time- and resource consuming than a longitudinal study, but are not able to determine the direction of the association. This is however, often not the case in genetic studies, as the studied genetic variants in our DNA are formed before the specific condition or trait is developed. Although the direction of the association is known, this does not imply causation (239).

In paper III, we used data from a prospective cohort study, where the outcome measures were continuous measures at a 12 months follow-up. With the longitudinal observation design it is possible to determine differences in exposures between individuals who develop a condition and who do not (108). Although we did not categorize patients into groups of chronic or no chronic pain or disability, our outcome measures at 12 months follow-up includes all ranges of pain and function affected by pain (0-10 VAS or 0-100% ODI), which makes it possible to differentiate between individuals.

### 5.2.2 Subjects and setting

Clinical and human experimental pain studies rely on healthy subjects to investigate abnormalities in different patient populations. However, there is no standardized definition of what is considered healthy (240). In paper I and paper II, healthy subjects were included if any chronic pain or somatic and mental illnesses were absent. This was however, self-reported. Subjects may consider themselves healthy if they are not diagnosed with medical conditions or their condition do not affect their daily life activities, but still have conditions that may influence their pain perception and thereby also the results of paper I and paper II (240). To assure inclusion of only healthy subjects, it is suggested to use screening checklists, questionnaires, medical examination and quantitative sensory testing before inclusion. However, selecting individuals with such a comprehensive approach may result in a group of "super-healthy" individuals, which is not representative for the general population. Studying "super-healthy" individuals may therefore increase the risk of detecting significant differences that are not clinically meaningful or related to the condition of interest. Paper II also included patients that had recovered from acute low back pain, and patients still in pain after an acute low back pain episode six weeks earlier. A heterogeneous sample consisting of healthy subjects and low back pain patients in different stages of recovery, may result in large variation and contributing to less power to detect significant differences and thereby increase the probability of a type II error. However, statistical analyses were conducted before

combining the groups to assure that the groups did not differ with regards to experimental pain test results and genetic variant allele frequencies. The study sample in paper III consisted of patients with LBP with radiculopathy. Stricter inclusion and exclusion criteria of LBP patients were used in paper III to assure a homogenous sample, and thereby more clinically relevant results.

There is always a risk of selection bias when enrolling volunteers to participate in studies, and maybe especially in experimental pain studies (241). In experimental pain studies, individuals who choose to participate may differ from individuals who choose not to participate in aspects regarding fear, risk-taking, level of self-confidence, tolerance of painful stimuli (241, 242). Similarly, patients who do not respond to follow-up assessments may differ from patients who do respond. In paper III, comparison of included patients and patients lost to follow-up were undertaken to describe such possible differences. Lost to follow-up could have been reduced with digital follow-up assessment, as postal questionnaires may be more inconvenient.
The advertising for recruitment of healthy volunteers in paper I and paper II took place at various educational institutions and the majority of the subjects were therefore students, which may reduce the generalizability to the general older population.

During the experimental pain assessments in paper I and paper II, the instructions, room temperature, placement of instruments and the experimenter clothes were standardized to prevent systematic variation attributable to the experimental setting. Acclimatization to the painful stimuli was performed to reduce the influence of motivation, attention and the subject's emotional state (243, 244). The subjects were informed of the testing procedures, but blinded for readouts from the stimulation instruments and study hypotheses. In paper I, the same female laboratory technician performed all experiments, which eliminates inter-rater variation (84, 245). In paper II, 4 different laboratory technicians (3 females) performed testing over the years from 2013 to 2018, which could lead to inter-rater variation. It was not possible to analyze inter-rater variation due to lack of data of which laboratory technicians that performed the experiments.

### 5.2.3 Outcome measures

In paper I, CPM effect was used as outcome measure. As recommended for studies assessing CPM, the CPM effect was reported using changes in both absolute values and percent changes (49). Due to different parameters used to calculate the CPM effect in the two protocols (VAS

vs. mA), the percent change CPM effect were used when the two protocols were compared. In paper II, pressure pain threshold, heat pain threshold, heat pain tolerance, CPM effect (absolute value and percent change), offset analgesia, temporal summation or secondary hyperalgesia were used as outcome measures. All the experimental tests for assessing pain used in the studies included in the present thesis are well established tests for assessing pain sensitivity and pain modulation. The tests provide relatively large effects and outcome measures with a continuous endpoint, which enables better differentiation between participants and increase the power of the studies. The specific methods of the test such as stimulation modality, duration, temperature increase/decrease rates, intensity level, etc. were chosen based on recommendations from the literature, the instrument manufacturer, or from previous experiences at our and our collaborators laboratories.

The outcome measure for paper III was back and leg pain intensity measured by NRS and function affected by pain measured by ODI. Back pain was used as the primary outcome measure to make the study more comparable with the GWAS and meta-analysis of chronic back pain, which was the basis of the selected genetic variants. Leg pain and function affected by pain was included as secondary outcome measures, as these are important symptoms for LBP with radiculopathy (246). Paresthesia and weakness are also rated as common symptoms of LBP with radiculopathy (247). Data of paresthesia and weakness were collected during clinical examination at baseline, but not collected at the 12 months follow-up assessment as this was mainly a follow-up with a postal questionnaire. We did not collect data from disease-specific questionnaires for LBP with radiculopathy, such as the Maine-Seattle Back Questionnaire (248), the Sciatica Bothersomeness Index or the Sciatica Frequency Index (249, 250). Therefore, the results of the study might not reflect the whole clinical picture of the included patients with LBP with radiculopathy.

For the outcome measures regarding back and leg pain, subjects were asked to rate their pain intensity for the past week. The relatively short timeframe decreases the probability of recall bias, but may be influenced by their present pain (88). It was not specified in the questionnaire whether the subjects should report average or maximum pain intensity for the past week. Fluctuations of pain may also decrease the precision of the estimated. Issues regarding recall bias, fluctuations of pain and the influence of maximal pain intensity, could have been avoided by using more specific formulations or daily questionnaires for a period of

time. However, one can assume that such bias is equally distributed across genotypes and thereby should not bias the results.

Because the follow-up assessment was 12 months after an acute episode of LBP with radiculopathy, pain and impaired function affected by pain would qualify as chronic symptoms if only duration of symptoms is used as the defining criteria. However, this definition does not take into account the body's inability to restore its function, which in regards to nerve compressions can range from weeks to years (251). Thus, we did not want to categorize the outcome measures into definite chronic or no chronic groups based on duration alone. In addition, there is no agreement on specific cut off-values for categorizing pain into recovery (157). For instance, recovery could only include patients who have no longer any symptoms (0 at a 0-10 NRS and 0 % at 0-100 % ODI), almost no symptoms (1-3 at a 0-10 NRS and 1-30 % at 0-100 % ODI), or patients who have had a clinical meaningful reduction in symptoms (change in outcome scores from baseline to 12 months). To avoid results affected by such additional defining criteria and to preserve as much statistical power as possible, we used continuous variables as primary and secondary outcome measures, and conducted sensitivity analyses with additional specified criteria. However, categories with the additional definition criteria resulted in very few patients in each genotype group. The results from these analyses were thereby underpowered and we considered it to be incorrect to present the results.

### 5.2.4 Power

The sample size calculation in paper I was based on previous studies from our laboratory (Lie et al., 2017, Nilsen, 2014), and we aimed to detect a difference in CPM effect between the two different CPM protocols. In comparison to clinical and experimental studies, sample sizes have received little attention in reliability studies (211). In regards to reliability, there is less agreement on the methods to calculate sample size, few available sample size calculators, and few studies who report performing sample size calculations. As a result, sample sizes are typically based on general recommendations and previous studies, which typically include 15-50 subjects (Lexell & Downham, 2005). The relatively small sample sizes in reliability studies compared to clinical studies may be related to the probability of false-positive results, as ICC values are criticized for being dependent of large sample sizes (de Vet et al. (2011). However, too small sample sizes often result in wide 95 % CI, which contribute to less accurate estimates.

Genetic markers found in association studies often have small effect sizes, and often explain only a small fraction of the genetic contribution to the diseases (31). However, candidate gene studies with sample sizes of 29 to 300 have reported associations for the selected genetic variants in COMT, 5HTTPLRP and OPRM1, where several studies report relatively large effect sizes in the context of genetic association studies(212, 214, 219, 224) . Nevertheless, one must keep in mind the high risk of publication bias in candidate gene studies. In paper II, we performed sample size calculations as described in Table 4, with a difference in pain score of 1.5 cm VAS between groups. In hindsight this approach has clear limitations, as it did not take into account correction for multiple tests and did not consider the selected genetic variant separately. The power calculation in paper II assumed a minor allele frequency of 0.2 – a rough average of the minor allele frequency in the Scandinavian population of the selected genetic variants in *COMT* and *OPRM1*, and previous results regarding the distribution of the combined genetic variant in *SLC6A4* (252). However, although the sample size in paper II is small compared to some association studies of clinical pain disorders, the sample size is relatively large compared to previous studies investigating association between these genetic variants and individual differences in experimental pain. That the present study with 356 subjects did not find evidence to support findings from previous studies of smaller sample size emphasizes the limitations of small experimental studies with a candidate gene approach and the need to replicate findings before they can be trusted. Nevertheless, due to the relatively small sample size in paper II, the present findings cannot be used to reject the hypothesis regarding a possible association with smaller effects sizes.

While GWAS based on large samples are best suited for the discovery of novel genetic associations, candidate gene studies may still have a role in the translation of GWAS findings into clinical utility. Due to the need for very large sample sizes in GWAS, the phenotyping and inclusion criteria are often less detailed, leading to a heterogeneous sample. Therefore, genetic associations identified in a GWAS may be driven by certain subgroups among the cases, in which the association effect sizes are larger than what is seen in the overall GWAS. The rationale for paper III was to investigate whether the previously identified GWAS SNPs are of clinical relevance for patients with low back pain (LBP) with persistent radiculopathy. We assumed that biomarkers used to tailor treatment or management of individual patients should have an effect size of 0.2 or more (Cohen's d). Paper III was therefore designed to find associations with an effect size of 0.2. If a common SNP does not have a measurable effect in a carefully phenotyped sample of 300 subjects, it is unlikely that these SNPs will have clinical

relevance as prognostic biomarkers. However, we cannot rule out true associations with smaller effect sizes or true associations with other phenotypes.

### 5.2.5 Statistical analyses

In paper I, a RM ANOVA was conducted to compare the CPM effect between two different CPM protocols in two separate sessions. RM ANOVA is a parametric analysis commonly used when each participant is exposed for two or more different conditions (253) and was therefore suitable for the study in paper I with normally distributed residuals and a continuous dependent variable. A RM ANOVA was preferred over a paired sample Student's t-test because 2 levels (two protocols and two sessions) were included as factors in the model (254). A linear mixed model is often considered better than RM ANOVA when analyzing repeated measures, as it is able to handle issues regarding non-normal residuals, missing data, clustering of subjects, complicated designs with more than one between-subjects factor and within-subjects factor, continuous variables as the time level and unbalanced repeated (255). As these issues were absent in the study in paper I, a linear mixed model would be unnecessarily complicated and not give any additional information to the results.

In paper I, the reliability of the CPM effect between two sessions was investigated with both relative and absolute reliability indices. Relative reliability for continuous variables is measured with correlation coefficients, Pearsons's *r* or intraclass correlation coefficient (ICC). Pearson's *r* is a measure of linear correlation between two sets of data, which provides a good indication of reliability between measures if only random errors exist, but do not take systematic errors into account. Parsons's *r* is therefore less critical than ICC and a high Parsons's *r* may occur even though the measurements vary (256). ICC is a modification of Parsons's *r* calculated by mean squares through analysis of variance, and reflects both the degree of correlation and agreement between measurements (257). ICC is therefore recommended as a reliability parameter for continuous variables (256), and therefore chosen over Parsons's *r*. There are several ICC equations and models, each appropriate to specific research designs (257). To correctly interpret the reliability and compare ICC values across studies, it is important to choose and report the appropriate equation. A two-way random effect model ($ICC_{2,1}$) with an absolute agreement definition for single measures was used in paper I. The $ICC_{2,1}$ equation was chosen due to an intra-rater design, where the rater was considered representative of a larger population of similar raters (257). This is the most used ICC equation (211) and the results are thereby more comparable across studies. The absolute

agreement model was chosen over a consistency model to include systematic differences as a source of variance, as the column variance is excluded from denominator variance in consistency analyses, making systematic differences irrelevant (258). The ICC was further calculated from a single measurement because the CPM effect was only measured once in each session. Although the use of ICC is commonly used to analyze reliability, it is not a sufficient measurement alone (211). ICC values tend to be lower in a homogenous group than in a heterogeneous group even though the difference in the outcome between sessions is similar in both groups (259). In paper I, the CPM effect ranged from -5.8 to 0.7 with the thermal protocol and -4.4 to 8.0 with the spinal reflex protocol, which suggests that the low ICC values are attributable to poor agreement between sessions and not because of a homogenous sample. High ICC values may also occur when subjects maintain their position in the sample across repeated measurements, even though the outcome has changed between sessions. ICC is also criticized for being dependent of the sample size, where high ICC values tend to occur when using large sample sizes (256). Because of these inherent problems with the ICC calculation, using ICC alone may lead to false conclusions regarding reliability, and it is therefore recommended to include measures of absolute reliability in test–retest reliability studies (256, 259).

There are numerous indices for assessing the absolute reliability, in which many of the indices only differs by their name or because of small differences in their equations (211). The Bland-Altman plots with 95 % limits of agreement and the related mean difference and $SD_{diff}$, is widely used in health research (256, 260), and were chosen to analyze the absolute reliability of the CPM effect between two separate sessions in paper I. For absolute reliability indices, there are no specific cut off-values for what is considered as acceptable values. The results have to be interpreted with knowledge about the outcome measure and what level is acceptable for practical use (211). As there is large variation in the methodology of assessing CPM, the acceptable level for minimal detectable change in CPM effect is not established. However, with psychophysiological approaches one could argue to use similar values of acceptable change as when using NRS or VAS to measure clinical pain, which are 1–2 points on a NRS or 1–2 cm on a VAS. In paper I, the CPM effect varied between sessions with more than 3 cm on the VAS with the psychophysiological protocol, which we therefore considered as poor reliability. The NWR assessed with the same instrument as in paper I has shown to fluctuate in average with approximately 2 mA over the course of 3 weeks (261), which

suggest that the CPM effect reported in paper I, have a relatively wide range of limits of agreement (–3.4 and 3.8 mA), especially considering the mean CPM effect of 0.4 mA.

In paper II and III, ANOVA, or the non-parametric alternative Kruskal-Wallis H test, were used to determine associations between the selected genetic variants and the different outcome measures as the dependent variables were categorical (253). Although genotypes are categories of alleles (Aa, AA or aa), genotypes are often treated as a continuous variable in genetic research as the risk of outcome often increases with the risk allele, and a regression model may therefore be appropriate. A linear regression also has the ability to tell which group that is different, while ANVOA gives an overall effect, with the possibility of post hoc tests to evaluate which groups that differs. However, the assumption of linearity was not fulfilled in either of the papers, and there was no difference between the groups, making the ANOVA a more suitable analysis.

## 5.3 Conclusions, implications and future perspectives

The present thesis explored individual differences in; 1) pain modulation with different assessments, and 2) experimental and clinical pain in relation to genetic susceptibility. We focused on a few biological factors of the biopsychosocial model hypothesized to explain some of the individual differences in pain.

The lack of a standardized CPM protocol impedes the use of CPM as a risk-, a predicting or a prognostic factor. Investigation of the magnitude and reliability of the CPM effect in different protocols is essential in order to discover standardized a protocol. Paper I is a contribution to getting a step closer towards using CPM as a tool for personalized pain treatment. In paper I we observed individual differences in CPM effect between two different CPM protocols, and that individuals had relatively high variations in CPM effect from one session to another session in both protocols. A larger CPM effect when using a CPM protocol with a thermal test-stimulus and a psychophysical outcome compared to using a CPM protocol with an electrical test-stimulus and a spinal reflex outcome, raises questions about whether CPM are more related to pain perception than nociception on a spinal level. The large variability observed in both protocols suggests that we should be cautions to use the two investigated CPM protocols in clinical decision making on an individual level, especially related to changes in CPM effect.

The physiological function of specific genes may explain some of the underlying mechanisms for individual differences in experimental pain. Paper II attempts to clarify conflicting results in the literature regarding three extensively studied genetic variants. In paper II we observed that genetic variation in three genes important for serotonin-, catecholamine- and opioid signaling, did not explain individual differences in experimental pain. The results may suggest that other factors are more important for individual differences in experimental pain than the selected genetic variants. However, the negative results may also be a reflection of small effect size of the selected genetic variants. Individual differences in experimental pain may still be dependent of the selected genetic variants when combined with each other and other genetic variants. We did not succeed to find associations previously reported in the literature, which illustrates the importance of replicating findings in genetic studies.

Identifying genetic variants that explain individual differences in clinical pain outcomes may explain the underlying mechanism for development of specific chronic pain conditions.

Genetic variants may also serve as prognostic biomarkers in a clinical setting. Paper III attempts to explore associations between genetic variants and LBP with radiculopathy. In paper III we observed that three genetic variants previously associated with back pain in a GWAS could not explain individual differences in clinical pain outcomes in patients with LBP with radiculopathy. It is possible that the genetic variants investigated are associated with LBP with radiculopathy, but the effect size is too small to detect a clinical measurable effect, suggesting that the selected genetic variants are not relevant as prognostic biomarkers at an individual level. The study emphasizes the importance of evaluating GWAS findings in smaller, but better described patient samples to translate such findings into clinical relevance.

Although the results from the present thesis do not allow practical implementation, it may carry information for further hypothesis about individual differences in pain modulation with different assessments, and individual differences in experimental and clinical pain in relation to genetic susceptibility. This may in turn contribute to a better understanding of pain and personalized pain treatment.

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ORIGINAL ARTICLE

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# Psychophysical or spinal reflex measures when assessing conditioned pain modulation?

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## Abstract

**Background:** Assessing conditioning pain modulation (CPM) with spinal reflex measures may produce more objective and stable CPM effects than using psychophysical measures. The aim of the study was to compare the CPM effect and test–retest reliability between a psychophysical protocol with thermal test-stimulus and a spinal reflex protocol with electrical test-stimulus.

**Methods:** Twenty-five healthy volunteers participated in two identical experiments separated by minimum 1 week. The thermal test-stimulus was a constant heat stimulation of 120 s on the subjects' forearm with continuous ratings of pain intensity on a 10 cm visual analogue scale. The electrical test-stimulus was repeated electrical stimulation on the arch of the foot for 120 s, which elicited a nociceptive withdrawal reflex recorded from the anterior tibial muscle. Conditioning stimulus was a 7°C water bath. Differences in the magnitude and test–retest reliability were investigated with repeated-measures analysis of variance and by relative and absolute reliability indices.

**Results:** The CPM effect was −46% and 4.5% during the thermal and electrical test-stimulus ($p < 0.001$) respectively. Intraclass correlation coefficient of 0.5 and 0.4 was found with the electrical and thermal test-stimulus respectively. Wide limits of agreement were found for both the electrical (−3.4 to 3.8 mA) and the thermal test-stimulus (−3.2 to 3.6 cm).

**Conclusions:** More pronounced CPM effect was demonstrated when using a psychophysical protocol with thermal test-stimulus compared to a spinal reflex protocol with electrical test-stimulus. Fair relative reliability and poor absolute reliability (due to high intraindividual variability) was found in both protocols.

**Significance:** The large difference in CPM effect between the two protocols suggests that the CPM effect relates to pain perception rather than nociception on the spinal level. Due to poor absolute intrarater reliability, we recommend caution and further research before using any of the investigated CPM protocols in clinical decision making on an individual level.

  

## 1 | INTRODUCTION

Assessment of endogenous pain modulatory function may carry a potential for stratification of treatment and follow-up of pain patients. One such measure is conditioned pain modulation (CPM), which assesses an individual's inherent ability to alter the central nervous system processing set up by a nociceptive stimulus (termed test-stimulus) in the presence of another nociceptive stimulus (termed conditioning stimulus) (Yarnitsky et al., 2010). CPM has been shown to be altered in several chronic pain conditions (Lewis, Rice, & McNair, 2012) and deficits may predict development of post-operative pain (Wilder-Smith, Schreyer, Scheffer, & Arendt-Nielsen, 2010; Yarnitsky et al., 2008) and treatment response (Nahman-Averbuch, Dayan, et al., 2016; Yarnitsky, Granot, Nahman-Averbuch, Khamaisi, & Granovsky, 2012). There is, however, a large variation in applied CPM methodology, which limits the generalization of conclusions for application in daily clinical practice (Pud, Granovsky, & Yarnitsky, 2009). Thus, there is a need for standardized and reliable methods to measure CPM (Yarnitsky et al., 2015).

CPM is usually assessed with psychophysical outcome measures, that is pain intensity ratings of supra-threshold stimuli or pain threshold assessment (Kennedy, Kemp, Ridout, Yarnitsky, & Rice, 2016; Pud et al., 2009), clearly involving subjective interpretation of the stimulus-induced percept. A systematic review suggests that CPM is a reliable measure, but the degree of reliability heavily depends on methodology (Kennedy et al., 2016). In a previous study, we reported large variability when using a protocol involving a thermal test-stimulus (Lie et al., 2017). Assessing CPM with standardized spinal reflex measures such as the nociceptive withdrawal reflex (NWR) elicited by electrical stimulations, may potentially be more reliable since such a measure may be less influenced by cognitive processes than psychophysical measures (Sandrini et al., 2005). One must, however, keep in mind that the withdrawal to the electrical stimulus is a reflex and not dependent of pain perception. Although not a painful outcome measure, it is commonly used as test-stimulus in CPM studies (Pud et al., 2009). The reliability of neuronal activity induced by an electrical test-stimulus has been investigated (Biurrun Manresa et al., 2014; Jurth, Rehberg, & Dincklage, 2014), but not compared with more commonly used psychophysical stimuli such as thermal test-stimuli. Therefore, the aim of this study was to compare the CPM effect and test–retest reliability between a CPM protocol using a thermal test-stimulus and a psychophysical outcome with a CPM protocol with an electrical test-stimulus and a spinal reflex outcome.

## 2 | METHODS

### 2.1 | Study design

This was an experimental crossover study comparing two CPM protocols with different test-stimuli (thermal vs. electrical) and different outcome measures (psychophysical vs. spinal reflex), but the same conditioning stimulus. A pretest was performed to familiarize subjects with the stimulations and pain intensity rating procedures, before the baseline test-stimulus was applied according to the protocol. After a 5-min break, the test-stimulus in parallel with a conditioning stimulus was applied. A 30-min break followed to eliminate carryover effects before the other protocol was carried out with the same procedure contralaterally (Figure 1). The experiment was repeated with a minimum interval of 7 days. The second session was identical to the first session in regards to randomization.

A computerized block-randomization for the order of protocol and the test side was conducted prior to the experiments. The subjects were informed of the testing procedure, but were not told whether the conditioning stimulus would influence the test-stimulus and were thus blinded for the study hypothesis. Subjects were also blinded for readouts from the stimulation instruments. A female experimenter (E.P) carried out all experiments. Instructions, placement of instruments, room temperature (21–23°C) and the experimenter's clothes were standardized.

A written informed consent was obtained prior to participation. The study was approved by the Regional committee

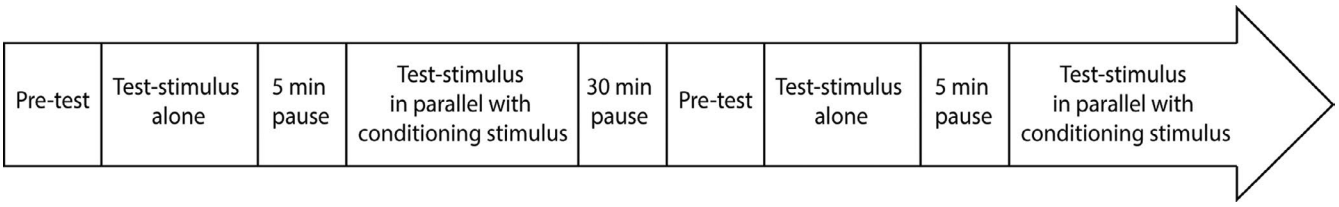


**FIGURE 1** Timeline of experiment. A pretest was performed to familiarize subjects with the stimulations and pain intensity ratings, before the test-stimulus (either thermal or electrical) was applied alone. After a 5-min break, a test-stimulus in parallel with the conditioning stimulus was applied. Thereafter, a 30-min break followed, before the protocol with the other test-stimulus was carried out with the same procedure contralaterally. The order of protocol and test-side was randomized prior to the experiment. An identical experiment was conducted after a minimum of 7 days

for medical and health research ethics (project no. 2010/2927) and conducted in accordance with the Declaration of Helsinki. Subjects received a gift certificate of NOK 500 for participation.

## 2.2 | Subjects

Men and women self-reported to be healthy and aged 18–45 years were recruited by advertisement at local hospitals and colleges/universities in Oslo, Norway. Exclusion criteria were as follows: chronic pain, somatic or psychiatric disease, headache for more than two days a month, hypertension (>140/90, assessed prior to the experiment after a 5 min rest), pregnancy (self-reported), breastfeeding, acquaintance with the experimenter and regular use of medication (including non-prescription pain killers) except oral contraceptives. Subjects were requested not to work nightshifts 48 hr before the experiment, not to consume alcohol or pain killers 24 hr before the experiment, or caffeine or tobacco the last hour before the experiment.

A priori power analysis based on previous studies from our laboratory (Lie et al., 2017; Nilsen, Olsen, Solem, & Matre, 2014) showed that 20 subjects were needed to detect a 10% difference in the absolute CPM effect in a paired Student's *t* test between the two protocols with a standard deviation of 1.5 cm on a 10 cm visual analogue scale (VAS, left end: 'no pain', right end: 'worst pain imaginable'), assuming a two-sided significance level of 5% and 80% power.

## 2.3 | Test-protocol

### 2.3.1 | Psychophysical outcome

Test-stimulus was contact heat stimulation induced by a 30 × 30 mm Peltier thermode (Medoc, Ramat Yishai, Israel) (baseline temperature: 32°C, increase rate: 2°C/s, decrease rate: 8°C/s) applied on the proximal volar aspect of the forearm with a constant temperature for 120 s (Figure 2a). The subjects continuously rated the pain intensity of the test-stimulus on a computerized 10 cm horizontal VAS by scrolling the wheel on a computer mouse. The stimulation site of the test-stimulus alone and the test-stimulus in parallel with the conditioning stimulus was not overlapping to avoid sensitization or habituation. The temperature of the test-stimulus was aimed to reflect pain intensity equal to approximately 6 cm on a 10 cm VAS. In order to find this temperature the following procedure was followed: An average of three tests of heat pain tolerance threshold tested with the methods of limits (baseline: 32°C, increase rate: 1°C/s) minus 2°C was calculated. The estimated temperature was tested with a 30 s heat stimulus positioned on the volar aspect of the opposite forearm. If the first 20 s was rated outside 4–9/10 cm VAS the temperature was adjusted accordingly.

### 2.3.2 | Spinal reflex outcome

Subjects were lying at a medical plinth with the back rest inclined 135° relative to the horizontal level, and a pillow under the knees assuring knee flexion of 45°. At stimulation sites existing hair was removed and the skin was lightly abraded and cleaned with sterilizing alcohol.

Electrocutaneous stimulation was applied through surface Ag/AgCl-electrode (30 × 22 mm, type Neuroline 720, Ambu A/S Denmark) placed on the medial aspect on the arch of the foot, and a large surface electrode (5 × 10 cm, Axelgaard, USA) placed on the dorsum of the foot just proximal to the toes (Figure 2b). This ensured that the stimulus was perceived in the arch of the foot. The electrodes were repositioned if subjects felt radiating sensation into the toes or on the dorsum of the foot. Recording electrodes were placed on the ipsilateral tibialis anterior muscle by three surface Ag/AgCl-electrodes (30 × 22 mm, type Neuroline 720, Ambu

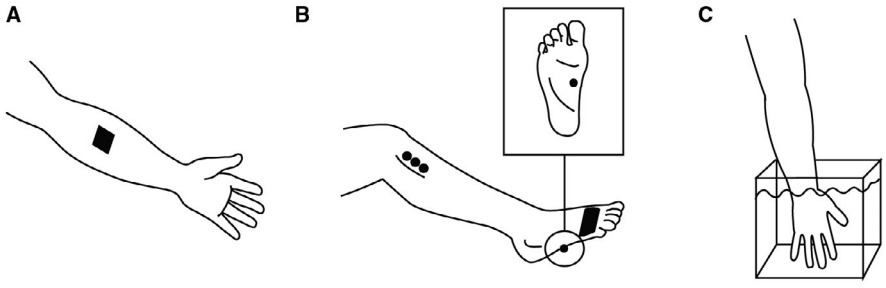


**FIGURE 2** Illustration of the test-stimuli and conditioning stimulus. (a) The thermal test-stimulus was contact heat stimulation applied on the proximal forearm with a constant temperature for 120 s. Pain intensity set up by the thermal test-stimulus was continuously rated on a computerized 10 cm visual analogue scale. (b) The electrical test-stimulus was induced by an electrode placed on the medial aspect on the arch of the foot, and a large electrode placed on the foot dorsum. Electromyographic reflex responses were recorded from the ipsilateral anterior tibial muscle by three electrodes. The nociceptive withdrawal reflex threshold was assessed when a peak *z* score was ≥12 in the poststimulus interval between 70 and 150 ms. (c) Conditioning stimulus was applied by immersing the hand contralateral to the test-stimulus in a 7°C water bath for 120 s or until the pain forced the subject to withdraw their hand from the water bath. The overall pain intensity of the conditioning stimulus was verbally rated immediately afterwards using a numerical rating scale from 0 to 10

A/S Denmark, one reference electrode) with an interelectrode distance of 2 cm. The skin was cleaned and abraded again if high impedance (>5 kΩ) occurred.

Trains of five 1 ms rectangular pulses (felt as a single stimulus) was delivered at 200 Hz with a 4 ms interpulse interval with Dolosys Paintracker (Dolosys GmbH, Berlin, Germany). Spinal reflexes measures may be difficult to standardize in clinical settings, and we wanted to use a commercial device which could be easy to implement in forthcoming clinical studies. Dolosys Paintracker is a commercial device which is easy to transport and set up and is therefore beneficial to use as a bedside-/point-of-care-test compared to other devices currently used to induce and measure electrical stimuli. The device is specifically developed to determine reflex thresholds continuously over a longer period of time.

The intensity of the electrical stimulus was the current needed to evoke a reflex threshold with interstimulus intervals randomized between 8 and 12 s to minimize stimulus predictability. The amplitude of the electromyographic (EMG) reflex responses to the electrical stimulations was converted to a peak *z*-score defined as the baseline-adjusted maximum divided by standard deviation of the EMG amplitudes before stimulation. The NWR threshold was defined as a peak *z*-score of ≥12 in the poststimulus interval of 70–150 ms (France, Rhudy, & McGlone, 2009). Electrical stimulations started at 1mA and increased with a rate of 0.5 or 1 mA until threshold was detected (minimum 8 values were needed for threshold calculation). After threshold detection, repeated stimulations were given for 120 s, resulting in a total of 10 electrical stimulations due to the interstimulus interval. Each stimulus was adjusted to be as close to a peak *z*-score of 12, for example if the previous stimulus elicited a large response, the intensity of the next stimulus was decreased. If this resulted in a threshold below a peak *z*-score of 12, the next stimulus was increased. Values of the three previous stimulations were used to determine if the intensity changed by 0.5 mA or 1.0 mA, which ensures precise threshold determination with the smallest possible steps (Instructions for use Paintracker, Dolosys GmbH). Subjects were told to relax their leg as much as possible, and were reminded to relax if muscle contractions in the leg (high EMG noise) were present between stimulations.

The overall level of unpleasantness and pain intensity of the electrical stimulations were rated verbally on a 0–10 numerical rating scale (NRS) (0 = 'no pain'/'no unpleasantness', 10 = 'worst pain imaginable'/'worst unpleasantness imaginable') after the test-stimulus was terminated.

## 2.4 | Conditioning stimulus

A 7°C circulating water bath (LAUDA Alpha RA8, LAUDA-Brinkman LP., USA) was used as conditioning stimulus in both protocols at the hand contralateral to the test-stimulus side (Figure 2c). With water up to the wrist, the hand was held wide open and steady for 120 s or until the pain forced the subject to withdraw the hand from the water bath. After 120 s, subjects were asked to rate the overall pain intensity of the conditioning stimulus on a 0–10 NRS (0 = 'no pain', 10 = 'worst pain imaginable').

## 2.5 | Data analysis

The CPM effect was defined as the difference in average pain intensity or NWR threshold between the test-stimulus alone and the test-stimulus in parallel with the conditioning stimulus. The CPM effect was also calculated as a percent change (CPM effect/test-stimulus alone × 100). The percent change in CPM effect was used when comparing the two protocols due to different parameters used to calculate the CPM effect. Additionally, subjects were categorized as CPM responders or non-responders. Subjects with decreased pain ratings during conditioning stimulus were defined as CPM responders in the protocol with the thermal test-stimulus, whereas subjects with increased reflex threshold were defined as CPM responders in the protocol with the electrical test-stimulus.

Statistical analyses were conducted using SPSS Statistics v. 21 (IBM, Armonk, NY). Findings with $p$ values ≤ 0.05 were regarded as significant. The distribution of data was assessed in preliminary analyses by a Shapiro–Wilk test and inspection of descriptive statistics, histograms, boxplots and Q–Q plots. These analyses did not indicate any extreme values or distributions that would affect the planned parametric analysis.

To determine whether a CPM effect was present, pain ratings or NWR threshold during the test-stimulus alone were compared with pain ratings or NWR thresholds during the test-stimulus in parallel with conditioning stimulus in paired sample Student's $t$ tests. Differences in CPM effect between the two protocols were estimated with repeated-measures analysis of variance (RM ANOVA), with session (levels: first session vs. second session) and protocol (levels: thermal protocol vs. electrical protocol) as factors.

Intraclass correlation coefficients with a two-way random-effect model ($ICC_{2,1}$) and absolute agreement definition for single measures were used to assess relative reliability (0.4: poor reliability; 0.4–0.59: fair reliability; 0.6–0.75: good reliability; 0.75: excellent reliability (Shrout & Fleiss, 1979). Bland–Altman plot and its related limits of agreement were used to assess the absolute reliability. Bias was calculated as the mean difference between the two sessions by subtracting the mean CPM effect in the first session from the second session, and then evaluated with a 1-sample Student's t test. 95% limits of agreement was calculated as mean difference ± 1.96 × SDdiff (SDdiff = *SD* of the mean difference).

## 3 | RESULTS

Twenty-eight subjects were included in the study. One subject did not participate in the second session for unknown reasons. One subject was excluded when previous participation in a similar study was revealed and one subject was excluded due to missing data because of technical issues. Thus, a total of 25 (11 females) were included in the analysis. Sample characteristics are presented in Table 1.

### 3.1 | CPM Effect

The mean CPM effect for the thermal protocol was −2.2 cm, representing a −46.0% decrease between pain ratings during test-stimulus alone and pain ratings during test-stimulus in parallel with the conditioning stimulus ($p < 0.001$) (Figure 3a). The mean CPM effect for the electrical protocol was 0.4 mA, representing a 4.5% increase between the NWR threshold during test-stimulus alone and NWR threshold during test-stimulus in parallel with conditioning stimulus ($p = 0.216$) (Figure 3b). The difference in CPM effect between the two protocols was significant ($p < 0.001$) (Figure 4) with a partial eta$^2$ effect size of 0.7. No significant differences in CPM effect was found between sessions ($p = 0.618$), and no interactions between sessions and protocols ($p = 0.949$). Post hoc analysis (RM ANOVA adjusted for changes in thresholds) showed, in contrast to the NWR thresholds, a significant CPM effect when using pain ratings (−32.5% decrease, $p = 0.002$, partial $\eta^2$ effect size 0.4) or unpleasantness ratings (−26.1% decrease, $p < 0.001$, partial $\eta^2$ effect size 0.5) of the electrical test-stimulus, comparing ratings during test-stimulus alone with ratings during test-stimulus in parallel with the conditioning stimulus (Table 4). A mean baseline noise of 0.6 μV was found with no significant difference between test-stimulus alone and during test-stimulus in parallel with conditioning stimulus, indicating low baseline muscle activity in both conditions.

**TABLE 1** Sample characteristics

| Variable | *n* (%/*SD*) |
|---|---|
| Sex, males, *n* (%) | 14 (56) |
| Age, years, mean (*SD*) | 24.1 (3.7) |
| BMI, kg/m$^2$,mean (*SD*) | 23.8 (2.0) |
| Relationship status | |
| Married/reg. partner, *n* (%) | 1 (4) |
| Partner, *n* (%) | 11 (44) |
| Single, *n* (%) | 13 (52) |
| Education | |
| Primary school 7–10 years, *n* (%) | 0 (0) |
| Vocational high school, *n* (%) | 4 (16) |
| General high school, *n* (%) | 11 (44) |
| College or university <4 years, *n* (%) | 10 (40) |
| College or university >4 years, *n* (%) | 0 (0) |
| Smoking, yes, *n* (%) | 2 (8) |
| Dominant hand, right, *n* (%) | 25 (100) |
| 120 s tolerance of conditioning stimulus | |
| During thermal test-stimulus 1st session, *n* (%) | 22 (88) |
| During thermal test-stimulus 2nd session, *n* (%) | 23 (92) |
| During electrical test-stimulus 1st session, *n* (%) | 24 (96) |
| During electrical test-stimulus 2nd session, *n* (%) | 24 (96) |
| CPM responders (CPM effect > 0) | |
| During thermal test-stimulus 1st session, *n* (%) | 24 (96) |
| During thermal test-stimulus 2nd session, *n* (%) | 25 (100) |
| During electrical test-stimulus 1st session, *n* (%) | 12 (48) |
| During electrical test-stimulus 2nd session, *n* (%) | 13 (52) |

Abbreviations: BMI, body mass index; CPM, conditioned pain modulation.

### 3.2 | Reliability

Detailed reliability values are shown in Tables 2 and 3. The ICC values of the CPM effect in both protocols were in the 0.40–0.59 range, which suggests fair relative reliability. Regarding absolute reliability, no bias was observed as there was no significant difference in mean difference between sessions in the protocol with thermal test-stimulus ($p = 0.631$) or the protocol with electrical test-stimulus ($p = 0.616$). Large limits of agreement were observed for the CPM effect in both protocols, which indicates large intraindividual differences between sessions (Figure 5).

## 4 | DISCUSSION

Our data showed significantly larger CPM effect using a protocol with a psychophysical outcome from using a thermal test-stimulus compared to a spinal reflex outcome using an electrical test-stimulus, where the latter protocol failed to detect a CPM effect. Fair relative reliability was observed for the CPM effect in both protocols. The absolute reliability indices in both protocols displayed good agreement in the mean CPM effect between the two sessions. However, high intraindividual variability was observed for both protocols.

### 4.1 | CPM Effect

The large difference in CPM effect between the two protocols (41.5%) indicate that the perceptual pain experience from a thermal test-stimulus is more prone to modulation during the conditioning stimulation than the NWR assessed by an EMG response to an electrical

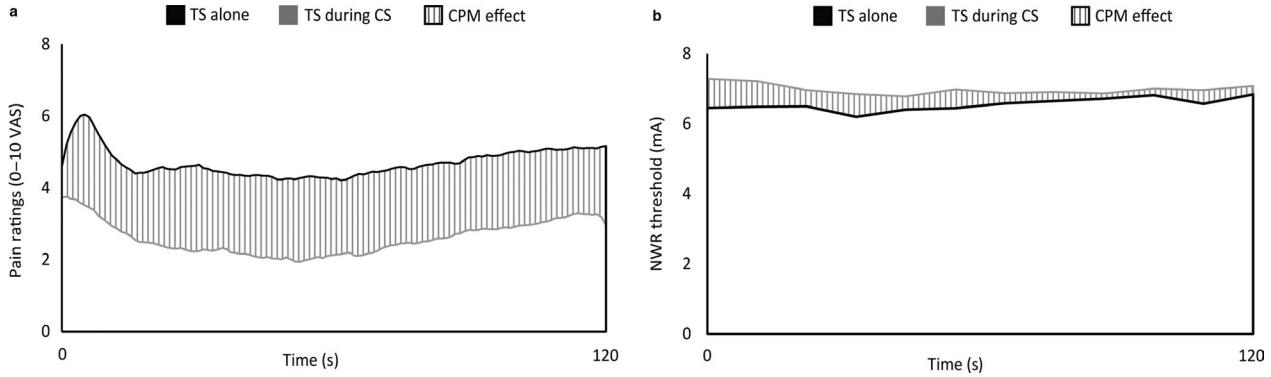


**FIGURE 3** The lines represent the average pain rating of the thermal test-stimulus (a) and the average NWR threshold of the electrical test-stimulus (b) during test stimulus alone (black) and during test stimulus in parallel with conditioning stimulus (grey). The difference between the test-stimulus-induced pain alone and the test-stimulus-induced pain during the conditioning stimulation (CPM effect) was significant ($p < 0.001$) for the former, but not for the latter ($p = 0.216$). CS, conditioning stimulus; CPM, conditioned pain modulation; NWR, nociceptive withdrawal reflex; TS, test-stimulus; VAS, visual analogue scale

test-stimulus. This is somewhat consistent with previous studies. Studies using 120 s heat test-stimulus report a CPM effect between −29 and −47% (Lie et al., 2017; Matre, Andersen, Knardahl, & Nilsen, 2016; Nilsen et al., 2014; Potvin et al., 2008; Tousignant-Laflamme, Page, Goffaux, & Marchand, 2008), whereas studies using electrical test-stimulus giving rise to a NWR, report a CPM effect between 11.5% and 40% (Biurrun Manresa et al., 2014; Bouhassira, Danziger, Attal, & Guirimand, 2003; Jurth et al., 2014; Sandrini et al., 2006). The somewhat higher CPM effect in other studies using an electrical test-stimulus in comparison to the result of this study may be due to different testing sites. The reflex in this study was elicited from the plantar surface of the foot and the response was measured from the anterior tibial muscle. The comparable studies stimulated the sural nerve trunk and recorded from the biceps femoris muscle. It is argued that sural nerve stimulation often is found intolerable resulting in a large number of failed tests, and that the currently employed set-up is less dependent on exact electrode positioning and demonstrates better test–retest reliability than sural nerve stimulation (Bouhassira et al., 2003; Jensen, Biurrun Manresa, & Andersen, 2015). Another difference, which could contribute to differences found in the CPM effect between this study and the comparable studies, is that they did not track the reflex threshold over a longer period of time (120 s).

In addition to a larger CPM effect, a larger proportion of CPM responders were observed using the protocol with thermal test-stimulus compared to the protocol with electrical test-stimulus. A possible explanation for lower CPM effect and fewer CPM responders when using electrical test-stimulus compared to thermal test-stimulus could be related to differences of the test-stimulus between the two protocols in regards to pain intensity, pain quality and the duration of the stimulus. The NWR threshold has been reported to be correlated with the subjective pain threshold (Sandrini et al., 2005). If this is the case, it is possible that a floor effect for the CPM effect for the electrical test-stimulus is present. The thermal test-stimulus was aimed to reflect a pain intensity of 6/10 on a VAS to prevent floor- or ceiling effects. One could argue that a supra-threshold, for example a NWR threshold × 1.5 instead of the NWR threshold may have resulted in a larger CPM effect in the protocol with electrical test-stimulus and also have more methodological similarity to the protocol with thermal test-stimulus. However, earlier studies have suggested that the NWR threshold is sufficient to detect a change in test-stimulus evoked by the conditioning stimulus CPM

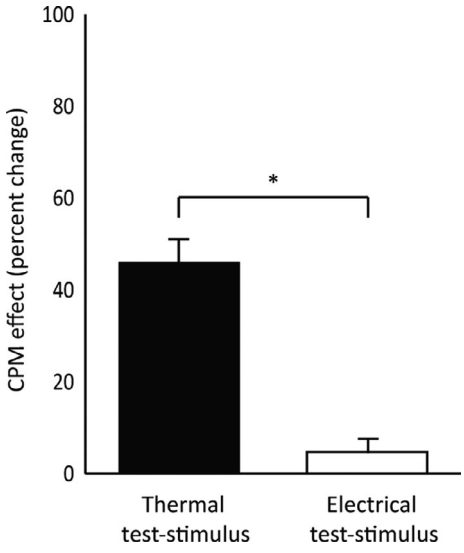


**FIGURE 4** There was a significant difference ($p < 0.001$) in percent change conditioned pain modulation (CPM) effect when using the thermal test-stimulus compared to the electrical test-stimulus. Error bars = standard error

**TABLE 2** Reliability indices for the protocol with thermal test-stimulus

| Variable | 1st session (mean, *SD*) | 2nd session (mean, *SD*) | Mean difference (95% LoA) | $ICC_{2,1}$ (95% CI) |
|---|---|---|---|---|
| Pain ratings of TS alone, 0–10 VAS | 4.7 (2.7) | 4.9 (2.5) | −0.2 (3.7–3.3) | 0.8 (0.5–0.9) |
| Pain ratings of TS during CS, 0–10 VAS | 2.4 (1.9) | 2.8 (1.9) | 0.4 (2.1–2.9) | 0.8 (0.6–0.9) |
| Pain ratings of CS, 0–10 NRS | 7.8 (2.0) | 7.6 (1.9) | −0.2 (2.6–2.3) | 0.8 (0.6–0.9) |
| CPM effect, 0–10 VAS | 2.3 (1.7) | 2.1 (1.5) | −0.2 (−3.2–3.6) | 0.4 (0.1–0.7) |

Abbreviations: CPM, conditioned pain modulation; CS, conditioning stimulus; $ICC_{2,1}$, intraclass correlation coefficients with a two-way random-effect model; LoA, limits of agreement; NRS, numerical rating scale; TS, test-stimulus; VAS, visual analogue scale.

**TABLE 3** Reliability indices for the protocol with electrical test-stimulus

| Variable | 1st session (mean, *SD*) | 2nd session (mean, *SD*) | Mean difference (95% LoA) | $ICC_{2,1}$ (95% CI) |
|---|---|---|---|---|
| NWR threshold during TS alone, mA | 7.0 (3.9) | 6.6 (2.9) | −0.4 (−4.9–4.2) | 0.8 (0.6–0.9) |
| NWR threshold TS during CS, mA | 7.3 (4.7) | 7.1 (3.4) | −0.2 (−6.6–6.3) | 0.7 (0.4–0.9) |
| Pain ratings of CS, 0–10 NRS | 7.4 (2.1) | 7.4 (1.8) | 0.0 (−1.9–2.0) | 0.9 (0.7–0.9) |
| CPM effect, mA | 0.3 (2.2) | 0.5 (1.3) | 0.2 (−3.4–3.8) | 0.5 (0.1–0.7) |

Abbreviations: CPM, conditioned pain modulation; CS, conditioning stimulus; $ICC_{2,1}$, intraclass correlation coefficients with a two-way random-effect model; LoA, limits of agreement; mA, milliampere; NRS, numerical rating scale; TS, test-stimulus.

effect and importantly, is more reliable than supra-threshold stimulation (Biurrun Manresa et al., 2014; Jurth et al., 2014). The NWR is commonly considered a proxy for nociception, due to its longer latency and higher threshold than the tactile reflex which first appears after an electrical stimulation (Willer, 1977). Still, it is still a possibility that the motor response may be contaminated by innocuous somatosensory processes, such as startle reactions and voluntary movements (although we attempted to reduce such influence by familiarization during pretests) or modulated by other types of descending control, for example emotions (Rhudy, Williams, McCabe, Russell, & Maynard, 2008) or attention/distraction (Bjerre et al., 2011).

The difference between the outcomes of the protocols may also be a result of different sites of stimulation, which can give rise to activity in different pain modulatory pathways. Two upper limbs are used in the protocol with the thermal test-stimulus, which may possibly reflect a segmental spinal inhibitory effect (although not necessarily limited to that). A combination of a lower limb and an upper limb is used in the protocol with electric test-stimulus, which may be more influenced by an ascending-descending modulatory activity. However, a recent study (Graven-Nielsen, Izumi, Petersen, and Arendt-Nielsen (2017) did not find any differences in CPM effect between upper and lower limb stimulation sites when using the same test-stimulus at different locations.

The large difference in CPM effect between the two protocols in our study raises questions as to the mechanisms of CPM. Larger CPM effect when the pain percept component is evaluated compared to when reflex processes are measured, suggests that CPM depends more on cognitive/evaluative aspects of the pain percept than on nociception. This theory is supported by our post hoc analysis where a significant CPM effect was observed when using pain ratings (−32.5%) or unpleasantness ratings (−26.1%) of the electrical test-stimulus. This result is in contrast to the traditional theory of a more limited neural system interaction, that is diffuse noxious inhibitory controls based on animal research, which is considered to rest on a spinal-supraspinal-spinal feedback loop. However, CPM in humans has shown to be highly influenced by supraspinal processes (Nahman-Averbuch, Nir, Nir, Sprecher, & Yarnitsky, 2016). Whether the modulation of pain perception found in this study is influenced by previous pain experiences, expectations, mood, attention or other modulatory influences from the central nervous system have not been embraced in this study protocol and needs to be addressed in future research.

A 7° cold water bath was chosen to induce pain ratings close to tolerance to ensure maximal CPM effect for all subjects, since conditioning stimulus with temperatures inducing higher pain intensity have shown to increase the CPM effect compared to temperatures inducing lower pain intensity or non-painful temperatures (Granot et al., 2008; Tousignant-Laflamme et al., 2008; Willer, Broucker, & Bars, 1989). However, it is desirable that the temperature and duration is tolerable enough to complete the conditioning stimulus according to protocol.

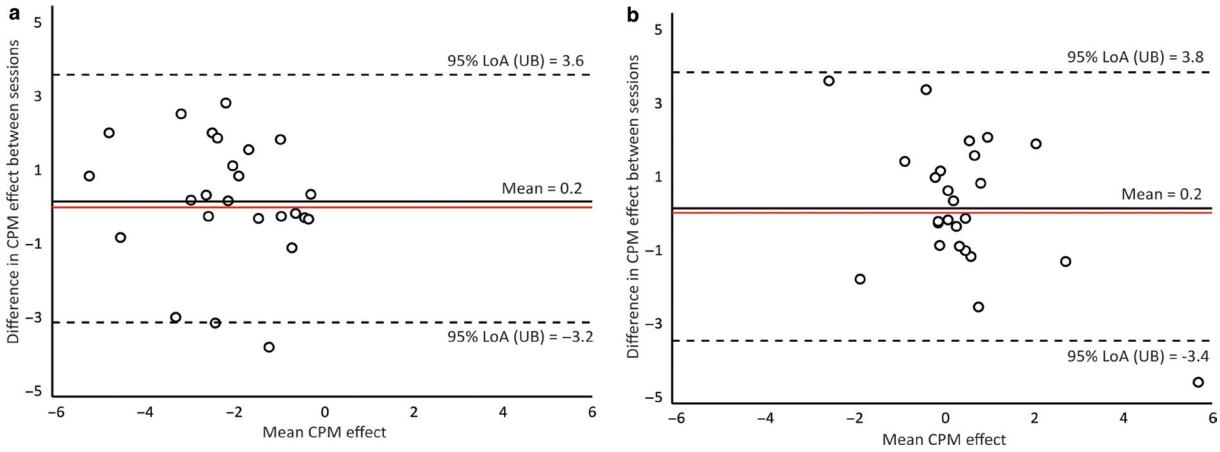


**FIGURE 5** Bland–Altman plot of the difference in CPM effect between sessions using the thermal test-stimulus (a) or the nociceptive withdrawal reflex as test-stimulus (b). Mean CPM effect are plotted against the difference between the two sessions. The red line represents no difference between the two sessions, whereas the black line represents the observed mean difference between sessions. The dotted lines represent 95% limits of agreement (upper boundary and lower boundary). CPM, conditioned pain modulation. LoA, 95% limits of agreement; UB, upper boundary; LB, lower boundary

**TABLE 4** Pain ratings and unpleasantness ratings of the electrical test-stimulus

| Variable | TS alone (mean, *SE*) | TS during CS (mean, *SE*) | CPM effect (mean, *SE*) | *p* value[a] | Partial $\eta^{2a}$ |
|---|---|---|---|---|---|
| Pain ratings, 0–10 NRS | 2.2 (0.4) | 1.5 (0.3) | 0.7 (0.4) | 0.003 | 0.3 |
| Unpleasantness ratings, 0–10 NRS | 3.8 (0.4) | 2.8 (0.3) | 0.9 (0.3) | <0.001 | 0.5 |

Abbreviations: CPM, conditioned pain modulation; CS, conditioning stimulus; NRS, numerical rating scale; SE, standard error.
[a] Repeated-measures analysis of variance, adjusted for change in nociceptive withdrawal reflex threshold.

The two protocols have many methodological differences that may affect the CPM effect and make comparison of the outcome of the two protocols difficult. First, the two protocols differ with respect to stimulation parameters such as type of stimulus, duration, stimulus intensity as well as pain intensity. Second, when increasing electrical stimulation intensity from 0, there is a range where stimulation is perceived as non-noxious. This means that the scales properties are not directly comparable. When the CPM effect is reported as a percent change for both methods, this may enhance the difference when comparing the CPM effect of the two protocols. However, both thermal and electrical protocols are commonly used to assess CPM and although it is difficult to find measures that are 100% comparable, the comparison of different protocols is important to find a golden standard protocol for CPM assessment.

## 4.2 | Test–retest reliability

Fair relative reliability was found in both protocols. In other studies using thermal test-stimuli, ICC values between 0.21 and 0.62 have been found (Gehling et al., 2016; Granovsky, Miller-Barmak, Goldstein, Sprecher, & Yarnitsky, 2015; Imai, Petersen, Morch, & Arendt Nielsen, 2016; Valencia, Kindler, Fillingim, & George, 2013; Wilson, Carvalho, Granot, & Landau, 2013). A recent systematic review concludes that differences in reliability heavily depend on stimulation parameters. However, in this study, the protocol with thermal test-stimulus was identical to a protocol used in a previous study conducted at our laboratory (Lie et al., 2017) which reported good relative reliability (ICC value 0.60). The difference in ICC values between our present (0.40) and our previous study highlights the variation in results despite identical protocols. It also emphasizes the limitations of ICC values as a measure of test–retest reliability. ICC strongly depends on the sample's heterogeneity; ICC values are lower in a homogenous group than in a heterogenous group although the difference in the outcome between sessions are the same in both groups (Atkinson & Nevill, 1998). High ICC values will also occur when subjects maintain their position in the sample across repeated measurements, even though the measurement (i.e. CPM effect) may have changed from session to session. Using ICC alone may lead to false conclusions regarding repeatability and it is therefore recommended to also include measures of absolute reliability in test–retest reliability studies (Atkinson & Nevill, 1998; Kennedy et al., 2016; de Vet, Terwee, Mokkink, & Knol, 2011). The relative reliability observed in studies using electrical test-stimulus is also conflicting; values between 0.26 (Biurrun Manresa et al., 2014) and 0.61 (Jurth et al., 2014) have been reported. A possible explanation for the poor reliability in our study may be different placement of the electrodes from session to session, even though we tried to prevent this

by standardized localization of the stimulation sites. In addition, the two sessions were not conducted at the same time during the day. Time of the day may to a minor degree influence the CPM effect (Aviram, Shochat, & Pud, 2015).

In both protocols, the bias between sessions was close to zero, suggesting absence of learning effects etc. However, large intraindividual variability was observed in both protocols, which indicate that neither of the protocols evokes a reliable CPM effect in healthy adults on an individual level. When it comes to comparing which of the two methods that is most reliable, the different outcome measured challenge the interpretation of the analysis. The level of absolute reliability depends solely on what is acceptable for practical use (Lexell & Downham", 2005). Considering the average CPM effect of 0.4 mA using the electrical test-stimulus, the wide range of limits of agreement (–3.4 to –3.8 mA) seems to constitute a genuine reliability problem. Levels of minimal detectable change when using NRS or VAS at 1–2 NRS points or 1–2 cm VAS is often considered acceptable. In this study, seven subjects (28%) showed a CPM difference between sessions of more than 2 cm on the VAS when using the thermal test-stimulus. Considering such a high proportion of subjects with high variability between tests, the implementation of CPM tests employed in this study is of limited value in clinical practice for stratification or prognostic purposes. However, whether CPM is a fluctuating parameter in healthy controls and a more stable parameter in patients suffering from pain conditions, should be addressed by future research before dismissing the applicability of the testing paradigm in clinical decision making.

## 5 | CONCLUSION

This study demonstrated a variable but fairly pronounced inhibitory CPM effect when the outcome measure is a psychophysical assessment of a thermal test-stimulus. Employing a spinal reflex outcome set up by a point-of-care device with electrical test-stimulus failed to demonstrate a CPM effect. Put together these results raise questions about the mechanisms involved in CPM testing. Fair relative reliability was observed for the CPM effect in both protocols, and poor absolute reliability was found in both protocols due to high intraindividual variability. One should be cautious to extrapolate the results from healthy adults to patients, and the large variability observed in our study calls for extended research in the clinical population before finally concluding on the applicability of CPM methodology in clinical decision making on an individual level.

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## CONFLICT OF INTERESTS

The authors have no conflict of interests to declare.

## AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design or analysis and interpretation of data as well as making intellectual contributions to the manuscript's content. All authors discussed the results and commented on the manuscript.

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II

**Original Experimental**

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# The association between selected genetic variants and individual differences in experimental pain

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**Abstract**

**Objectives:** The underlying mechanisms for individual differences in experimental pain are not fully understood, but genetic susceptibility is hypothesized to explain some of these differences. In the present study we focus on three genetic variants important for modulating experimental pain related to serotonin (SLC6A4 5-HTTLPR/rs25531 A>G), catecholamine (COMT rs4680 Val158Met) and opioid (OPRM1 rs1799971 A118G) signaling. We aimed to investigate associations between each of the selected genetic variants and individual differences in experimental pain.

**Methods:** In total 356 subjects (232 low back pain patients and 124 healthy volunteers) were genotyped and assessed with tests of heat pain threshold, pressure pain thresholds, heat pain tolerance, conditioned pain modulation (CPM), offset analgesia, temporal summation and secondary hyperalgesia. Low back pain patients and healthy volunteers did not differ in regards to experimental test results or allelic frequencies, and were therefore analyzed as one group. The associations were tested using analysis of variance and the Kruskal-Wallis test.

**Results:** No significant associations were observed between the genetic variants (SLC6A4 5-HTTLPR/rs25531 A>G, COMT rs4680 Val158Met and OPRM1 rs1799971 A118G) and individual differences in experimental pain (heat pain threshold, pressure pain threshold, heat pain tolerance, CPM, offset analgesia, temporal summation and secondary hyperalgesia).

**Conclusions:** The selected pain-associated genetic variants were not associated with individual differences in experimental pain. Genetic variants well known for playing central roles in pain perception failed to explain individual differences in experimental pain in 356 subjects. The finding is an important contribution to the literature, which often consists of studies with lower sample size and one or few experimental pain assessments.

**Keywords:** experimental pain; genetic susceptibility; pain modulation; pain sensitivity.

## Introduction

Assessments of experimental pain are assumed to be of clinical value in management of pain patients, but the underlying mechanisms for individual differences in experimental pain are not fully understood and needs to be better addressed. Assessments of experimental pain may include tests for pain sensitivity, e.g. pain threshold and pain tolerance, or tests that assess the dynamic function of pain modulation, e.g. conditioned pain modulation (CPM), offset analgesia, temporal summation and secondary hyperalgesia.

Increased pain sensitivity has been associated with numerous pain disorders [1–3] and is regarded as one of the characteristics in central sensitization of the nervous system [4]. CPM represents reduced pain perception of a painful stimulus (test-stimulus) when a second painful stimulus (conditioning stimulus) is inflicted and is

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assumed to measure inhibitory pain modulation [5]. CPM has also been associated with pain disorders [6] and has been shown to predict the development of pain [7–9] and treatment response [10, 11]. Offset analgesia is another measure of inhibitory pain modulation, where a disproportionate decrease in pain perception is seen after a small decrease in stimulus intensity [12]. Similar to CPM, offset analgesia has been associated with pain disorders [13–15]. Tests that reflect central sensitization in pain disorders are temporal summation, which represents an increase in pain perception despite no change in stimulation intensity [16, 17] and secondary hyperalgesia, which is present if the tissue beyond an area of tissue damage (primary hyperalgesia) becomes hypersensitive [18, 19].

One of the underlying mechanisms for individual differences in experimental pain is genetic susceptibility. Many genetic variants are assumed to be important for modulating pain perception, but genetic variants related to serotonin (5-HT), catecholamine and opioid signaling have been of particularly interest and extensively studied due to their physiological function [20]. However, results from studies examining association between these variants and individual differences in experimental pain in humans are conflicting [21–25] and more studies are needed to elucidate whether these genetic variants can explain individual differences in experimental pain. Therefore, the present study aimed to investigate associations between each of the selected genetic variants; *SLC6A4* 5-HTTLPR/rs25531 A>G, *COMT* rs4680 Val158Met and *OPRM1* rs1799971 A118G, and individual differences in experimental pain.

# Methods

## Study design

The present study used data from a prospective cohort study of acute low back pain patients admitted to a hospital (n=232) [26, 27]. The present study was a cross-sectional study using socio-demographic data assessed through questionnaires, blood samples collected at hospital admission, and data from experimental pain testing performed six weeks after hospital admission. Similar data have been collected from healthy volunteers participating in studies at the same laboratory as the low back pain patients (n=124) [27–29]. The present study combined data from the low back pain patients and healthy volunteers.

A written informed consent was obtained prior to participation. The study was approved by the regional committee for medical and health research ethics in Norway (project number: 2010/2927, 2012/1108) and was conducted in accordance with the Declaration of Helsinki. Healthy volunteers received a gift certificate of NOK 250 for participation.

## Study population

Patients were recruited from the Department of Neurology at Oslo University Hospital in Norway between January 2013 and June 2018. Inclusion criteria were age 18 years or older, acute low back pain with or without radiating pain, pain rated ≥4 on an 11 point numeric rating scale (NRS) (0='no pain', 10='worst pain imaginable'). Healthy volunteers were recruited by advertisement at local hospitals and colleges/universities in Oslo, Norway. Inclusion criteria were men and women self-reported to be healthy, aged 18–60 years. Exclusion criteria for patients and healthy volunteers were non-Caucasian heritage (mother or father), inability to understand spoken or written Norwegian, not currently working, previous or current alcoholism or substance abuse, regular use of neuroleptics and tricyclic antidepressants, pregnancy, breastfeeding, psychiatric or somatic diseases making the person unsuitable for inclusion, spinal fracture, malignancy, infection, cauda equina syndrome, rapidly progressive neurologic deficits or chronic pain defined as pain rated ≥4 on an NRS for ≥3 month in the last two years.

## Experimental pain testing

The experimental pain testing procedure consisted of standardized tests for sensitivity (pressure pain thresholds, heat pain threshold and heat pain tolerance) and for pain modulation (CPM, offset analgesia, temporal summation and secondary hyperalgesia). Subjects were blinded to the study hypothesis and readouts from the stimulation instruments. A pretest was performed to familiarize subjects with the stimulations and pain intensity rating procedures. Subjects continuously rated the pain intensity on a computerized 10 cm horizontal visual analog scale (VAS) (left end (0 cm): 'no pain', right end (10 cm): 'worst pain imaginable') by scrolling the wheel on a computer mouse in all constant heat stimulations if not otherwise described. See supporting information TableS1 for instrumental details of the different tests.

**Pressure pain threshold:** To assess pressure pain threshold, the experimenters manually increased pressure (5 N/s) on muscle trapezius with a 1 $cm^2$ pressure algometer (AlgoMed, Medoc, Ramat Yishai, Israel). The subjects rated their pain by moving a knob along a 10 cm VAS on a box. The left side of the line represented 'no pain', and the right side line represented 'worst pain imaginable'. The subjects were instructed to not move the knob until pain was first experienced. Assessments were performed bilaterally and an average value of the two assessments was used in the analyses.

**Heat pain thresholds and tolerance:** Heat pain threshold and heat pain tolerance were assessed with gradually increasing the temperature during stimulation on the distal volar aspect of the right forearm with a 30 × 30 mm Peltier thermode (baseline temperature: 32 °C, increase: rate 2 °C/s, decrease rate: 8 °C/s) (Pathway model ATS, Medoc, Ramat Yishai, Israel). When assessing heat pain threshold, subjects were instructed to stop the increase in temperature by clicking on a computer mouse when they felt the first sensation of pain. When assessing heat pain tolerance, subjects were instructed to click on the computer mouse when they could not tolerate the increasing temperature any longer. The temperature was automatically stopped

at 52 °C for safety reasons. If the subject did not reach its threshold before 52 °C, this temperature was noted as the threshold. The tests were repeated three times and an average value was used in the analyses.

**Pain6 calculation:** A temperature aimed to reflect pain intensity equal to approximately 6 cm on 10 cm VAS (Pain6) was used during the tests for pain modulation. In order to estimate the Pain6 temperature for each individual, 2 °C was subtracted from an average of three tests of pain tolerance (see section heat pain thresholds and tolerance). The estimated temperature was thereafter tested with a 30 s heat stimulus with a 30 × 30 mm Peltier thermode (baseline temperature: 32 °C, increase rate: 1 °C/s, decrease rate: 8 °C/s) (Pathway model ATS, Medoc, Ramat Yishai, Israel) on the left thenar eminence. If the first 20 s of the stimulation was rated outside 4–9 cm on a 10 cm VAS, the temperature was adjusted accordingly.

**Conditioned pain modulation:** To assess CPM, a baseline test-stimulus was applied, followed by a 5-min break, before an identical test-stimulus was applied in parallel with a conditioning stimulus. The test-stimulus was a constant heat stimulation from a 30 × 30 mm Peltier thermode (baseline temperature: 32 °C, increase rate: 1 °C/s, decrease rate: 8 °C/s) (Pathway model ATS, Medoc, Ramat Yishai, Israel) with Pain6 temperature for 120 s on the right forearm. The conditioning stimulus was the opposite hand immersed in a 7 °C circulating water bath (LAUDA Alpha RA8, LAUDA-Brinkman LP., New Jersey, USA) with water up to the wrist and the hand held wide open for 120 s or until the pain forced the subject to withdraw the hand from the water bath. After 120 s, subjects were asked to rate the pain intensity of the conditioning stimulus on a 0–10 NRS. To avoid sensitization or habituation of the stimulated area, the area of the baseline test-stimulus and the test-stimulus in parallel with conditioning stimulus was not overlapping. Fifty of the healthy volunteers were part of a subproject and were randomized in regards to stimulation arm. A CPM effect was defined as the difference in average pain intensity between the test-stimulus alone and the test-stimulus in parallel with the conditioning stimulus. The CPM effect was also calculated as a percent change (CPM effect/test-stimulus alone × 100).

**Offset analgesia:** Two trials with heat stimulation with a 30 × 30 mm Peltier thermode (baseline temperature: 32 °C, increase rate: 1 °C/s, decrease rate: 8 °C/s) (Pathway model ATS, Medoc, Ramat Yishai, Israel) on the right forearm were used to assess offset analgesia. One trial had 30 s constant Pain6 temperature, while the other trial consisted of a three-temperature paradigm; first, heat stimulation was applied with Pain6 temperature for 5 s (T1). Next, the temperature was increased by 1 °C and kept constant for 5 s (T2) before the temperature returned to the initial temperature and kept constant for 20 s (T3). The stimulated area of the two trials was not overlapping to avoid sensitization or habituation of the stimulation area. The order and position of the trials were randomized, and the trials were separated by a 2-min break. Offset analgesia was calculated as the difference in pain ratings between T3-T2 during the three-temperature paradigm compared to the same time interval in the constant stimulation.

**Temporal summation:** Temporal summation was assessed by heat stimulation with a 30 × 30 mm Peltier thermode (baseline temperature: 32 °C, increase rate: 1 °C/s, decrease rate: 8 °C/s) (Pathway model ATS, Medoc, Ramat Yishai, Israel) on the right forearm with a constant Pain6 temperature for 120 s, except for 50 of the healthy volunteers who were part of a subproject and were randomized in regards to the stimulation arm. Temporal summation was defined as an increase in pain ratings (>0 cm) on a 10 cm VAS from the start (30–40 s) to the end (110–120 s) of the stimulation.

**Secondary hyperalgesia:** A 5-min heat stimulation of 45 °C with a 30 × 30 mm Peltier thermode (baseline temperature: 32 °C, increase rate: 1 °C/s, decrease rate: 8 °C/s) (Pathway model ATS, Medoc, Ramat Yishai, Israel) was used to create an area of primary hyperalgesia in the center of the volar aspect of the left forearm. After a 2-min break, a von Frey filament (Touch-Test TM Sensory Evaluator, Stoelting, Illinois, USA) was used to map the area of secondary hyperalgesia. The filament was pressed against the skin at 90° angle until the filament bowed, starting at a 5–6 cm distance from the heat stimulation area and repeated every 0.5 cm with 3–4 s intervals in eight directions 45° towards the heat stimulation area. The order of the directions was randomized. Subjects were instructed to look away from the arm and indicate when a prick had a clear change in sensation. This point was then marked with a colored pencil. After all directions were tested, the markings were transferred on to transparency film. The area of secondary hyperalgesia was extracted and calculated with Engauge Digitizer Software, version 10.8.

## Genotyping

Blood samples were obtained in 4 ml EDTA tubes and frozen at –80 °C until DNA extraction was performed with QIAamp DNA Blood Kit (n=326) or QIAGEN Autopure LS (n=30) according to the manufacturer's protocol (QIAGEN, Valencia, CA, USA). The genetic variants genotyped were *SLC6A4* 5-HTTLPR/rs25531 A>G, *COMT* rs4680 Val158Met and *OPRM1* rs1799971 A118G. Genotypes were determined using fast quantitative real time polymerase chain reactions (qPCR) (Gene Amp, PCR System 9700, Applied Biosystems, California, USA). PCR amplifications were performed with 384-well plates containing genomic DNA, TaqPath ProAmp Master Mix and TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA) (see supporting information TableS2 for details of the genotyping). Negative controls containing water only were included in every run. Samples with undetermined genotypes were re-genotyped. The overall genotype call rate was 98%.

Regarding the *SLC6A4* 5-HTTLPR/rs25531, we performed gel electrophoresis to determine the long (529 bp) and short (486 bp) allele. Fragments were visualized with ultraviolet light after 2 h separation at 80 V in TAE buffer on a 2.5% agarose gel (MetaPhor™ Agarose, Lonza, Cologne, Germany), containing GelRed (Biotium Inc, California, USA). As previously described [30], the *SLC6A4* 5-HTTLPR and *SLC6A4* rs25531 were divided into three groups; low ($S_A/S_A$), medium ($SL_G$, $L_A/L_G$, $SL_A$) or high ($L_A/L_A$) 5-HTT expression types.

## Statistical analysis

Statistical analyses were conducted using SPSS Statistics version 25 (IBM, Armonk, NY). The distribution of sample characteristics and experimental pain test results were assessed in preliminary analyses by a Shapiro–Wilk test for normality and inspection of descriptive statistics, histograms, boxplots, and Q-Q plots.

Sample size calculations showed that with a two-sided significance level of 5 and 80% power, 228 subjects were needed to detect a 10% difference in pain scores between genotypes with a standard deviation of 20 cm on a 10 cm visual analogue scale (VAS, left end: 'no pain', right end: 'worst pain imaginable'), assuming a genetic variant is present in 20% of the population. When offset analgesia and secondary hyperalgesia were added to the test protocol, new sample size calculations were made based on a standard deviation of 17, resulting in 168 subjects needed to detect a difference. No difference in individual differences in experimental pain have been observed between our two samples of low back pain patients and healthy volunteers ([27] and unreported studies), so to increase our sample size we chose to combine low back pain patients and healthy volunteers in the association analysis. To ensure that findings was due to associations between genetic variance and individual differences in experimental pain, low back pain patients and healthy volunteers were tested for systematic differences in sample characteristics, individual differences in experimental pain and genotype distributions. Similar comparison were done between patients who had almost or fully recovered from the acute back pain (defined as <3 VAS at the six weeks follow-up) and patients still in a pain state when the experimental tests were performed (defined as leg pain ≥3 VAS at the six weeks follow-up). Independent sample Student's t-test was used for normally-distributed variables, Mann-Whitney U-test was used for variables with non-normal distribution, and Chi-square or Fisher's exact test was used for categorical variables.

Paired sample Student's t-test was used to determine if there was a CPM effect, temporal summation, and offset analgesia. Analysis of variance (ANOVA) or the non-parametric alternative Kruskal-Wallis test was used to determine the association between the selected genetic variants and individual differences in experimental pain.

Since earlier studies have shown that *OPRM1* A118G may be sex-specific [31–33], a multivariate ANOVA was performed to investigate interactions between *OPRM1* A118G and sex. Findings with p-values<0.01 were regarded as significant for all statistical analyses due to multiple testing.

# Results

## Sample characteristics

In total 356 subjects (232 back pain patients and 124 healthy volunteers) were genotyped and pain tested (Figure 1). Sample characteristics and gene frequencies are presented in Table 1, with details in Supplementary Table 3. Low back pain patients and healthy volunteers did not differ with regards to sample characteristics or distribution of genotypes, except for age (p<0.001), body-mass index (BMI) (p<0.001) and diastolic blood pressure (p<0.001) (Supporting information TableS3). No differences were found in sample characteristics and distribution of genotypes between patients with leg pain VAS <3 or VAS ≥3, except for age (p=0.002) and education (p=0.002) (Supporting information TableS3).

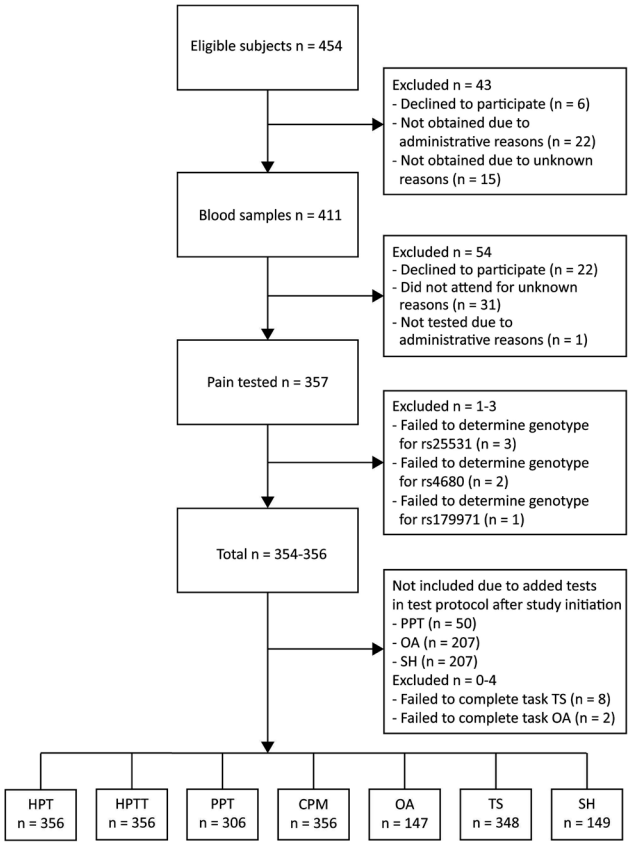


**Figure 1:** Flowchart.
PPT, pressure pain threshold; HPT, heat pain threshold; HPTT, heat pain tolerance; CPM, conditioned pain modulation; OA, offset analgesia; TS, temporal summation; SH, secondary hyperalgesia.

**Table 1:** Sample characteristics.

| Variable | n | Value |
|---|---|---|
| Sex (males), n (%) | 357 | 208 (58.3) |
| Age (years), median (IQR) | 357 | 35 (26–45) |
| Education (>12 years), n (%) | 357 | 321 (89.9) |
| Left handed, n (%) | 346 | 38 (10.6) |
| BMI (kg/m2) mean (SD) | 351 | 25.2 (3.6) |
| Systolic blood pressure (mmHg), mean (SD) | 357 | 123.3 (13.3) |
| Diastolic blood pressure (mmHg), mean (SD) | 357 | 74.7 (9.7) |
| Current smoker, n (%) | 354 | 44 (12.3) |
| 5HTTLPR/rs25531 *(SLC6A4)*, MAF | 354 | 0.3 |
| Val158Met *(COMT)*, MAF | 355 | 0.4 |
| A118G *(OPRM1)*, MAF | 356 | 0.1 |

IQR, inter quartile range; SD, standard deviation; MAF, minor allele frequency.

## Experimental pain tests

Results from the assessments of pain modulation are presented in Figure 2A–D. There was a significant difference between pain ratings during baseline test-stimulus

and pain ratings during test-stimulus in parallel with the conditioning stimulus (effect size=−2.5, SD=1.7, p<0.001), representing a CPM effect of −48.9%. In the offset analgesia paradigm, there was a significant difference between pain ratings during T3-T2 in the constant stimulation and pain ratings during T3-T2 in the three-temperature paradigm (effect size=−0.5, SD=1.8, p<0.001). Temporal summation of pain during constant heat stimulation was found, as there was a significant difference between pain ratings at the start and at the end of the constant heat stimulation (effect size=0.6, SD=2.1, p<0.001). For none of the experimental pain tests did test results differ between patients and healthy volunteers, or between patients with leg pain VAS<3 or VAS≥3 (Supplementary Table 3).

## Genetic associations

There were no significant associations between any of the selected genetic variants, *SLC6A4* 5-HTTLPR/rs25531 A>G, *COMT* rs4680 Val158Met and *OPRM1* rs1799971 A118G and individual differences in experimental pain assessed with pressure pain threshold, heat pain thresholds, heat pain tolerance, CPM, offset analgesia, temporal summation or secondary hyperalgesia (Figure 3 and Table 2). No significant interaction was found between *OPRM1* A118G and sex in regards to individual differences in experimental pain (p=0.575).

# Discussion

In the present study, we found no association between the selected genetic variants, *SLC6A4* 5-HTTLP/ rs25331 A>G, *COMT* Val158Met or *OPRM1* A118G and individual differences in pressure pain threshold, heat pain threshold, heat pain tolerance, CPM, offset analgesia, temporal summation or secondary hyperalgesia. To our knowledge, the present study is one of the largest candidate gene study investigating associations between the selected genetic variants and individual differences in experimental pain. The present study is also the first to explore the association between the selected genetic variants and offset analgesia and secondary hyperalgesia.

The serotonin transporter (5-HTT), encoded by the *SLC6A4* gene, plays a central role in the uptake of serotonin in the synaptic cleft. A length polymorphism (5-HTTLPR) in the promoter region of *SLC6A4* results in two common variants; a short (S) and a long (L) allele [34–36]. The S allele leads to reduced 5-HTT expression, which may influence 5-HT signaling [37, 38]. In addition, a single nucleotide polymorphism (SNP) rs25531 A>G in the same promoter region is also associated with reduced 5-HTT expression [39]. Previous studies have shown a relationship between *SLC6A4* 5-HTTLPR/rs25531 A>G and individual differences in experimental pain, where the low 5-HTT expression type typically is associated with lower heat pain threshold [23, 40], and impaired CPM [24], but one study

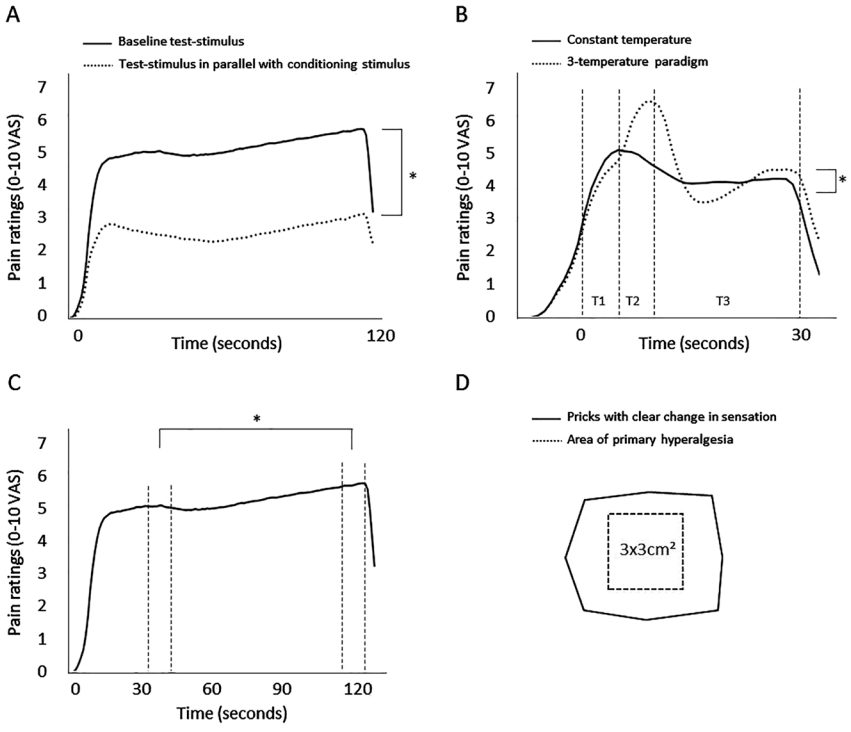


**Figure 2:** Results from the experimental pain assessments of pain modulation.
(A) A conditioned pain modulation (CPM) effect was present, with a decrease in pain ratings of the test-stimulus during conditioning stimulus (p<0.001), (B) Offset analgesia was present with a larger decrease in pain ratings in the three-temperature paradigm than in the constant paradigm (p<0.001), (C) Temporal summation of pain was observed with a significant increase in mean pain ratings during the continuous heat pain stimulation (p<0.001). The vertical lines marks the time periods that was compared, (D) Illustration of the area of secondary hyperalgesia for 97 of 149 subjects. In 52 of the subjects the direction of the transparency film, which the markings were transferred to, was unknown and could not be used in the illustration.
VAS, visual analog scale.

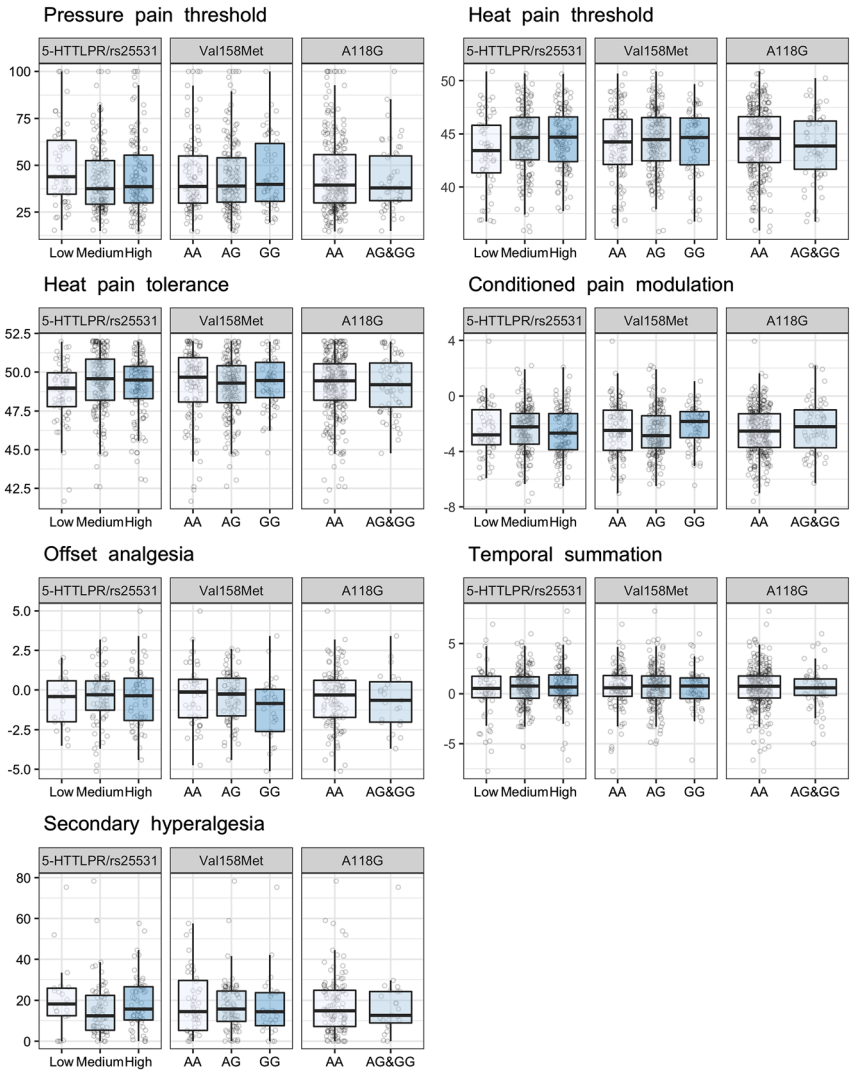


**Figure 3:** Associations between the selected genetic variants and individual differences in experimental pain. Findings with p-values≤0.01 were regarded as significant. There was no significant association between the selected genetic variants and individual differences in experimental pain.

observed higher heat pain thresholds for the low expression type [41]. The present study did not show significant evidence to support these studies, but may point in the direction of a possible association between low expression type and lower heat pain threshold (p=0.03) and heat pain tolerance (p=0.04) due to the observed trend. Consistent with our results, some studies have shown no relationship between the *SLC6A4* 5-HTTLPR/rs25531 A>G and individual differences in experimental pain [25, 42–44]. A possible explanation for the diverse findings in the literature can be use of different test parameters when assessing experimental pain. Another explanation for conflicting results could be related to the complexity of the serotonergic system. A high concentration of serotonin in the synaptic cleft may impact the nearby postsynaptic 5-HT receptors, which results in increased signaling, or it may impact the presynaptic autoreceptors, which results in an increase of negative feedback and thereby decrease signaling [35]. However, this may depend on the location of localization of the 5-HTT relative to the 5-HT autoreceptors [45, 46]. Serotonin is also regulated by the seven different groups of 5HT-receptors, which mediate both excitatory and inhibitory neurotransmission.

Catechol-O-methyltransferase (COMT) encoded by the *COMT* gene, is an enzyme that promote degradation of catecholamines (dopamine, epinephrine, and norepinephrine). The SNP rs4680 G>A causes a substitution of the amino acid valine (Val) to methionine (Met) at codon 158, and reduces enzyme activity which results in higher levels of catecholamines [47]. The relationship between *COMT* Val158Met and individual differences in experimental pain has been studied in numerous animal and human pain models. Results are somewhat conflicting, with some studies reporting that the Met allele is associated with lower pressure pain thresholds, heat pain threshold, and temporal summation [21, 48–51], while other studies find

**Table 2:** Associations between the selected genetic variants and individual differences in experimental pain. Findings with p-values<0.01 were regarded as significant.

| Variant (gene) | Genotype | n | Mean (SD)/median (IQR) | $r^2/\chi^2$ | p-Value | n | Mean (SD)/median (IQR) | $r^2/\chi^2$ | p-Value | n | Mean (SD)/median (IQR) | $r^2/\chi^2$ | p-Value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Pressure pain threshold[a] | | | | Heat pain threshold[b] | | | | Heat pain tolerance[a] | | |
| 5-HTTLPR/rs25531 *(SLC6A4)* | Low[c] | 51 | 43.9 (34.3–65.5) | 4.3 | 0.12 | 63 | 43.3 (3.4) | 33.7 | 0.03 | 63 | 49.0 (47.7–50.0) | 6.7 | 0.04 |
| | Medium[d] | 141 | 37.5 (29.2–52.5) | | | 169 | 44.4 (3.1) | | | 169 | 49.6 (48.2–50.9) | | |
| | High[e] | 114 | 38.6 (29.8–55.7) | | | 124 | 44.5 (2.9) | | | 124 | 49.5 (48.3–50.4) | | |
| Val158Met *(COMT)* | AA | 98 | 38.7 (29.7–55.4) | 1.0 | 0.79 | 114 | 44.1 (3.2) | 2.9 | 0.82 | 114 | 49.7 (48.0–51.0) | 2.2 | 0.53 |
| | AG | 152 | 38.9 (30.1–54 0) | | | 176 | 44.4 (3.0) | | | 176 | 49.3 (48.0–50.4) | | |
| | GG | 54 | 39.8 (30.4–63.5) | | | 64 | 44.2 (3.2) | | | 64 | 49.5 (48.3–50.7) | | |
| A118G *(OPRM1)* | AA | 253 | 39.4 (29.9–56.0) | 0.3 | 0.60 | 294 | 44.3 (3.1) | 14.7 | 0.21 | 294 | 49.4 (48.2–50.5) | 0.1 | 0.75 |
| | AG + GG | 53 | 37.9 (30.8–55.4) | | | 62 | 43.8 (3.1) | | | 62 | 49.2 (47.7–50.6) | | |
| | | | Conditioned pain modulation[b] | | | | Offset analgesia[b] | | | | | | |
| 5-HTTLPR/rs25531 *(SLC6A4)* | Low[c] | 63 | −2.4 (1.8) | 1.0 | 0.73 | 21 | −0.7 (1.6) | 0.6 | 0.84 | | | | |
| | Medium[d] | 169 | −2.4 (1.7) | | | 69 | −0.5 (1.7) | | | | | | |
| | High[e] | 123 | −2.6 (1.7) | | | 56 | −0.5 (1.9) | | | | | | |
| Val158Met *(COMT)* | AA | 114 | −2.4 (1.9) | 7.1 | 0.07 | 43 | −0.3 (2.0) | 4.3 | 0.25 | | | | |
| | AG | 176 | −2.6 (1.7) | | | 73 | −0.4 (1.6) | | | | | | |
| | GG | 63 | −2.1 (1.5) | | | 29 | −1.1 (1.9) | | | | | | |
| A118G *(OPRM1)* | AA | 293 | −2.5 (1.7) | 4.7 | 0.21 | 123 | −0.5 (1.8) | 0.4 | 0.73 | | | | |
| | AG + GG | 62 | −2.2 (1.9) | | | 23 | −0.6 (1.8) | | | | | | |
| | | | Temporal summation[b] | | | | Secondary hyperalgesia[a] | | | | | | |
| 5-HTTLPR/rs25531 *(SLC6A4)* | Low[c] | 62 | 0.2 (2.6) | 7.8 | 0.16 | 21 | 18.2 (12.3–26.0) | 4.2 | 0.12 | | | | |
| | Medium[d] | 163 | 0.7 (1.8) | | | 71 | 12.4 (5.3–22.6) | | | | | | |
| | High[e] | 122 | 0.8 (2.0) | | | 57 | 15.7 (10.4–26.6) | | | | | | |
| Val158Met *(COMT)* | AA | 113 | 0.6 (2.2) | 0.3 | 0.97 | 44 | 14.5 (5.2–30.3) | 2.3 | 0.52 | | | | |
| | AG | 169 | 0.7 (2.0) | | | 75 | 15.7 (9.1–24.4) | | | | | | |
| | GG | 63 | 0.6 (1.9) | | | 29 | 14.4 (7.2–23.9) | | | | | | |
| A118G *(OPRM1)* | AA | 288 | 0.7 (2.1) | 0.8 | 0.67 | 126 | 14.9 (7.1–24.9) | 0.1 | 0.75 | | | | |
| | AG + GG | 59 | 0.5 (2.0) | | | 23 | 12.6 (7.6–24.2) | | | | | | |

SD, standard deviation; IQR, inter quartile range; $r^2$, Analysis of variance (ANOVA) R-squared value; $\chi^2$, Kruskal-Wallis test chi-squared value.

[a]Presented with median (IQR) and Kruskal-Wallis $\chi^2$.

[b]Presented with mean (SD) and ANOVA $r^2$.

[c]Low 5-HTT expression type ($S_A/S_A$).

[d]Medium 5-HTT expression type ($SL_G$, $L_A/L_G$, $SL_A$).

[e]High 5-HTT expression type ($L_A/L_A$).

an opposite effect [22] or no association with individual differences in experimental pain [52], consistent with the present study's results. However, the observed trend between *COMT* Val158Met and CPM (p=0.07) in the present study, may suggest a possible association between the Met allele and impaired CPM. The inconsistencies in the literature may be due to different sample selection [51, 53] and sample sizes, different choice of experimental tests or different tests protocols [22, 54].

Opioid signaling is regulated by the μ opioid receptor encoded by the *OPRM1* gene. The SNP rs1799971 A>G causes a substitution of the amino acid asparagine to aspartic acid at codon 40, and removes a putative N-linked glycosylation site in the receptor, which may affect the function of the receptor [55, 56]. The G allele in *OPRM1* A118G has been associated with higher pressure pain thresholds [57, 58], which is in contrast with the present study. Similar to the present study, some studies found no relationship between *OPRM1* A118G and heat pain threshold and pressure pain threshold [59–62]. The conflicting results could potentially be explained by sex-differences. An asparagine to aspartic amino acid substitution in *OPRM1* A118G affects the glycosylation site of the receptor, which is important for cellular processes such as receptor folding, sorting, expression and ligand binding [63]. The level and type of glycosylation have shown to be different between female and male mice [33, 64], and some human studies have shown opposite effects of *OPRM1* A118G in men and women [31, 32, 65]. For this reason we also analyzed the interaction of *OPRM1* A118G and sex in regards to individual differences in experimental pain, but no such interactions were found.

## Strength and limitations

The present study investigated pain sensitivity as well as anti- and pro-nociceptive functions of the pain system. We chose tests which have relatively large effects, with the outcome measure as a continuous endpoint, which enables differentiation between subjects and increase the power of the study. To date, there is no gold standard for assessing the dynamic function of the pain system. When using a genetic model to predict individual differences in experimental pain, one assumes that experimental pain response is a stable trait. However, results of experimental pain assessments has been shown to be influenced by psychological and environmental factors [66], and the reliability of the different tests range from poor to good depending on the methodology of the tests as well as statistics [67, 68]. Further research should establish gold standards for assessing experimental pain, which will likely lead to more consistent results between studies, and improve the chances to identify genetic risk factors.

The present study sample was heterogeneous, consisting of both healthy volunteers, patients that had recovered from acute low back pain, and patients still in pain after an acute low back pain episode six weeks earlier. Combining experimental data from patients and healthy volunteers are potentially problematic, but could be done because the groups did not differ with regards to experimental pain test results and genetic variant allele frequencies. In a genetic association study, factors such as age and sex are not considered potential confounders, since they do not affect the genetic variants, but sample heterogeneity can lead to reduced power, contributing to our negative results [69]. Although the sample size of the present study is small compared to association studies of clinical pain disorders, the sample size is relatively large compared to studies investigating association between the same selected genetic variants and individual differences in experimental pain. That the present study with 356 subjects does not find evidence to support findings from studies of smaller sample size emphasizes the limitations of experimental studies with a candidate gene approach and the importance of replication of findings before conclusions can be reached. Several experimental tests are often used in experimental pain studies, however few are adjusting for multiple testing. In the present study, a stricter significance level was used to decrease the probability of making a type I error, but remain power to detect significance for the experimental tests that typically are highly correlated.

We did only investigate the effect of three genetic variants and cannot exclude that other polymorphisms in these or other genes affect individual differences in experimental pain. However, the genetic variants studied were carefully selected based on their physiological function as well as previous research demonstrating their relationship to individual differences in experimental pain.

In conclusion, the selected genetic variants, *SLC6A4* 5-HTTLPR/rs25531 A>G, *COMT* rs4680 Val158Met and *OPRM1* rs1799971 A118G, were not associated with individual differences in experimental pain.

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III

## Title

Low back pain with persistent radiculopathy; the role of the genetic variants in the genes *SOX5*, *CCDC26/GSDMC* and *DCC*


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## Disclosures

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**Abstract**

In a recently published genome-wide association study (GWAS) chronic back pain was associated with three loci; *SOX5, CCDC26*/*GSDMC* and *DCC*. This GWAS was based on a heterogeneous sample of back pain disorders, and it is unknown whether these loci are essential for low back pain (LBP) with persistent radiculopathy. Thus, we examine if LBP with radiculopathy 12 months after an acute episode of LBP with radiculopathy is associated with the selected genetic variants; *SOX5* rs34616559, *CCDC26/GSDMC* rs7833174 and *DCC rs4384683*. In this prospective cohort study, subjects admitted to a secondary health care institution due to an acute episode of LBP with radiculopathy, reported back pain, leg pain, and Oswestry Disability Index (ODI), were genotyped and followed up at 12 months (n = 338). Kruskal-Wallis H test showed no association between the genetic variants and back pain, leg pain or ODI. In conclusion, LBP with radiculopathy 12 months after an acute episode of LBP with radiculopathy, is not associated with the selected genetic variants; *SOX5* rs34616559, *CCDC26/GSDMC* rs7833174 and *DCC rs4384683.* This absent or weak association suggests that the genetic variants previously associated with chronic back pain are not useful as biomarkers for LBP with persistent radiculopathy.

**Keywords**

Low back pain, Lumbar radiculopathy, Genetic susceptibility, Biomarkers, Candidate gene study

## 1. Introduction

Low back pain (LBP) is the leading cause of years lived with disability in Western countries (1) and represents a large economic burden (2-4). Although the majority of patients with LBP recover within a few weeks, a significant number of patients develop chronic LBP (5). One common condition of LBP is radiculopathy, in which the patient experiences pain and/or paresthesia in the distribution of the lumbar spinal nerve due to a nerve root compression (6). Radiculopathy is more persistent, severe, has a less favorable outcome and consumes more health resources than LBP (7), and may therefore account for a considerable part of the socioeconomic burden of LBP. Thus, to reduce the socioeconomic burden of LBP, better treatment, monitoring and prevention of LBP with radiculopathy are needed. Genetic susceptibility is assumed to play an important role for LBP with radiculopathy (8-11). The use of such genetic information in personalized medicine holds great promise to improve health care. A genome- wide association study (GWAS) is a suitable approach to identify new associations between diseases and genetic variants (12). However, genetic association studies with a candidate gene approach using smaller, but better described, patient samples with a detailed phenotyping is often necessary to evaluate the clinical value of findings from GWASs, such as whether genetic variants can be used as prognostic biomarkers on an individual level. In the first reported GWAS meta-analysis of chronic back pain with 158.000 individuals three loci were identified in or near the genes *SOX5, CCDC26*/*GSDMC* and *DCC* (13). However, this GWAS comprised a heterogeneous sample of chronic back pain disorders, and it is unknown whether these loci are essential for LBP with persistent radiculopathy. Thus, we aimed to examine if LBP with radiculopathy 12 months after an acute episode of radiculopathy is associated with the selected genetic variants; *SOX5* rs34616559, *CCDC26/GSDMC* rs7833174 and *DCC rs4384683*.

## 2. Methods

### Design

The present study used data from a prospective cohort study of subjects which required admission to a secondary health care institution due to an acute episode of LBP with radiculopathy. Questionnaires, clinical and radiological examination, and blood samples were obtained at admission. Questionnaires were obtained 12 months after admission, either by post or at a follow-up assessment. If the 12-month questionnaire was not returned within two weeks, subjects were reminded by mail and/or phone. The institutions' health care personnel assisted in inclusion, data collection and follow-up of patients, but the subjects were treated independently of the study with lumbar surgery and/or conservative treatment.

A written informed consent was obtained prior to participation. The study was approved by the regional committee for medical and health research ethics (project number: 2013/1060) and conducted in accordance with the Declaration of Helsinki.

**Subjects**

Subjects were recruited from three hospitals in Norway; 1) the Neurological Department at Oslo University Hospital between January 2013 and June 2018 (n = 301), 2) a secondary health care back clinic at the Østfold Hospital Trust between January 2014 and May 2016 (n = 86), and 3) Department of Neurosurgery at St. Olavs hospital, Trondheim between January 2015 and May 2016 (n = 50). Inclusion criteria were age 18 years or older and the presence of acute radiculopathy defined as acute LBP with radiating pain with dermatomal distribution corresponding to radiological findings of lumbar nerve root compression. Exclusion criteria were non-Caucasian heritage (mother and father), unable to understand spoken and written Norwegian, previous or current alcohol or substance abuse, pregnancy, breastfeeding, spinal fracture, malignancy, infection, cauda equina syndrome, rapidly progressive neurologic deficit, or psychiatric, somatic or chronic disorder making the subject unsuitable for inclusion. Subjects recruited from the Østfold Hospital Trust also excluded subjects with prior surgery at the same disc level, lumbar fusion and spinal stenosis. Data from the Department of Neurosurgery at St. Olavs hospital were collected through the Norwegian Registry for Spine Surgery (NORspine), which only included surgical patients.

**Measures**

Self-reported questionnaires measured sex (man/woman), age (years), education (≤ 12 years/ > 12 years), height (cm), weight (kg), smoking habits (yes (included occasionally)/no (included former smoker)), daily medication use (yes/no), pain intensity in the past week on a 0-10 numeric rating scale (NRS, 0 = 'no pain', 10 = 'worst pain imaginable'), function affected by pain (Oswestry Disability Index, ODI), pain duration (> 3 months/ < 3 months) and recovery (pain intensity < 3 NRS 12 months after admission/pain intensity ≥ 3 NRS 12 months after admission). Treatment data (surgical/conservative) was collected by study personnel.

The primary outcome measure for LBP with radiculopathy was back pain intensity 12 months after an acute episode of LBP with radiculopathy. Due to the diversity of clinical symptoms in radiculopathy, two secondary outcome measures were also used; leg pain intensity and ODI 12 months after an acute episode of LBP with radiculopathy. ODI contains ten sections which regard intensity of pain, the influence of pain on the ability to take care of oneself, lift, walk, sit, sleep quality, sexual function, social life and travel. Each section is scored on a scale from 0 to 5 with 0 representing no disability and 5 representing severe disability. An ODI score was calculated by dividing the summed score by the total possible score which is then multiplied by 100 (0 = no disability and 100 = maximum disability possible).

**Genotyping**

Blood samples were obtained at hospital admission in 4 ml EDTA tubes and frozen at -80 ˚C until DNA extraction was performed with QIAamp DNA Blood Kit according to the manufacturer's

protocol (QIAGEN, Valencia, CA, USA). Due to no predesigned TaqMan SNP genotyping assay for *SOX5* rs12310519, it was replaced with a genetic variant in high LD (rs34616559, $r^2$=0.95 and D'=1.00). Genotypes were determined using fast quantitative real time polymerase chain reactions (qPCR) (Gene Amp, PCR System 9700, Applied Biosystems, California, USA). PCR amplifications were performed using 384-well plates containing 2.25 mL genomic DNA, 2.5 mL TaqPath ProAmp Master Mix (Thermo Fischer scientific Inc, Waltham, MA USA) and 0.25 mL predesigned TaqMan SNP genotyping assay (Thermo Fischer scientific Inc, Waltham, MA USA) and included initialization for 10 minutes at 95˚C, followed by 40 cycles of 15 seconds at 95˚C and 1 minute at 60˚C. Negative controls containing water were included in every run. Approximately 10% of the samples were re-genotyped with a concordance rate at 100%. Samples with undetermined genotypes were re-genotyped. The overall genotype call rate was 98%.

**Statistical analysis**

Statistical analyses were conducted using SPSS Statistics version 25 (IBM, Armonk, NY). The distribution of the data and residuals was assessed in preliminary analyses by a Shapiro–Wilk test and inspection of descriptive statistics, histograms, boxplots, and Q-Q plots.

Sample size calculations showed that 246 subjects were needed to detect a difference between the three genotypes in each genetic variant with an effect size of 0.2, a two-sided significance level of 5%, 80% power and an assumption that the risk genotype is present in 5% of the population.

Subjects who responded to the follow-up questionnaire at 12 months were compared with subjects who did not respond with independent sample Student's t-test for normally-distributed variables, Mann-Whitney U test for variables with non-normal distribution, and Chi-square for categorical variables. A Wilcoxon Signed Rank test was used to determine differences between baseline and 12 months for back pain, leg pain and ODI. Missing data were not imputed. The number of subjects with missing genetic data is presented in figure 1, and the number of missing data for the outcome measures is presented in table 1.

Kruskal-Wallis H test was used to analyze the association between each genotype (3 allele groups) of the selected genetic variants (*SOX5* rs34616559, *CCDC26/GSDMC* rs7833174 and *DCC rs4384683*) and each outcome measure (back pain, leg pain and ODI) due to non-normal distribution of the residuals of the outcome measures, and violation of the linearity assumption.

## 3. Results

**Sample characteristics**

Sample characteristics are presented in table 1. Of the 501 eligible subjects, blood samples were not obtained for 64 subjects due to administrative reasons or because subjects declined to participate. Of the 436 subjects included in the study, 49.1 % were admitted to a secondary health care institution due

to acute LBP with radiculopathy, and 50.9 % were admitted due to an acute worsening of their already persistent LBP with radiculopathy (defined as back and/or leg pain duration > 3 months). There was a significant improvement from baseline to 12 month in regards of back pain ($p<0.001$), leg pain ($p<0.001$) and ODI ($p<0.001$). However, 38.9 % had back/leg pain intensity ≥ 3 NRS 12 months after an acute episode of LBP with radiculopathy. In addition to conservative treatment, 62 % of the subjects were offered surgical treatment.

The dropout rate at 12 months was 22.5 % (Fig. 1). There was no significant difference between subjects who responded to the follow-up questionnaire at 12 months and subjects who did not, except for age (43.8 years vs. 38.6 years, $p<0.001$) and smoking habits (15.2 % smoker vs. 26.6 % smoker, $p = 0.010$) (table 1).

**Genetic associations**

There was no statistical significant association between the primary outcome measure, back pain intensity 12 months after an acute episode of radiculopathy, and the selected genetic variants (Fig. 2). There were also no significant associations between the secondary outcome measures, leg pain intensity and ODI 12 months after an acute episode of radiculopathy, and the selected genetic variants (Fig. 2).

## 4. Discussion

The recently published GWAS meta-analysis mentioned in the introduction of this paper suggested that the genetic variants *SOX5* rs34616559, *CCDC26/GSDMC* rs7833174 and *DCC* rs4384683 may be linked to chronic back pain (13). Since a considerable proportion of the sample used in this GWAS may suffer from radiculopathy, we hypothesized that these genetic variants also would have an impact on subjects with LBP with radiculopathy. However, the present study found no associations between LBP with radiculopathy 12 months after an acute episode of LBP with radiculopathy and the selected genetic variants. Thus, the present study indicates that the genetic variants reported in the GWAS meta-analysis of chronic back pain (*SOX5* rs34616559, *CCDC26/GSDMC* rs7833174 and *DCC* rs4384683) are not of prognostic value in a clinical setting for subjects admitted to a secondary health care institution for an acute episode of LBP with radiculopathy.

*SOX5* is a member of the *SOX* family of transcription factors which are critical for chondrocyte differentiation during embryonic development as well as notochord development (14-16), thus *SOX5* may play an important role in the formation of the spine and the intervertebral discs. *SOX5* has also been associated with cartilage and osteoarthritis in animal studies (17-19), but not in human cartilage (20). In the GWAS meta-analysis of chronic back pain, chronic back pain was most strongly associated with rs12310519 in the *SOX5* gene (13), which may indicate that *SOX5* plays a role in the underlying mechanisms for other chronic back pain conditions than those causing radiculopathy since we did not discover a similar association in the present study. Two GWA studies of LBP with

radiculopathy support the present study findings as they do not find any SNP's in the *SOX5* gene associated with LBP with radiculopathy (9, 11).

*GSDMC* (Gasdermin C) is a member of the *GSDM* family and contains a conserved two-domain structure (N-terminal and C-terminal domains). When the N-terminal domain is released, it possesses pore-forming activity, which results in loss of cell membrane integrity and release of inflammatory mediators and thereby causes inflammatory cell death (21). *GSDMC* is associated with differential methylation patterns in osteoarthritis-related cartilage and subchondral bone cartilage (22, 23). CCDC26 is a long non-coding RNA gene, which modulates retinoic acid, which in turn increases apoptosis (controlled cell death). Absence of apoptosis may result in cancer, while excessive apoptosis may result in a cell death of vital cells causing neurodegenerative diseases (24). The present study did not support the hypothesis that the genetic variant rs7833174 located between *CCDC26* and *GSDMC* is associated with LBP with radiculopathy 12 months after an acute episode of LBP with radiculopathy. However, *CCDC26*/*GSDMC* rs7833174 have been associated with lumbar microdiscectomy (9) and lumbar spinal stenosis (25), and a different variant *GSDMC* rs77681114 have been associated with lumbar disc herniation (26). Lumbar micro discectomy is a common treatment for lumbar disc herniation (27) and both lumbar disc herniation and lumbar spinal stenosis may cause radiculopathy (7). The difference between the present study and the other studies investigating genetic associations for conditions that may cause or be associated with LBP with radiculopathy, is that they use healthy controls as the control group, while the present study only use subjects with LBP with radiculopathy that has recovered. *CCDC26*/*GSDMC* rs7833174 may be associated with LBP with radiculopathy when compared to healthy controls, but may not be able to differentiate between different severities of LBP with radiculopathy.

*DCC* (Deleted in Colorectal Cancer) encodes the netrin-1 receptor, which acts as a tumor suppressor when not bound to netrin-1, and as an axon guidance when bound to netrin-1. Netrin-1/*DCC* interactions are involved in pain processing, as it have shown that increased *DCC* expression can cause sprouting of myelinated afferent fibers in the spinal dorsal horn, which may result in mechanical allodynia in animal models (28). Netrin-1/*DCC* may also play a role in underlying mechanisms for chronic discogenic back pain, as degenerated intervertebral discs have increased expression of netrin-1 compared to healthy control discs (29). However, the present study does not support the hypothesis that this genetic variant is relevant for LBP with radiculopathy 12 months after an acute episode of LBP with radiculopathy. The two GWA studies of LBP with radiculopathy support the present study findings as they do not find any SNP's in the *DCC* gene associated with LBP with radiculopathy (9, 11).

**Strength and limitations**

Findings from GWA studies give valuable information, but due to the need of large sample sizes, the phenotyping and inclusion criteria are often less detailed, leading to a heterogeneous sample. Follow-up studies in cohorts with detailed phenotyping and strict inclusion criteria such as the present study are needed to translate findings into clinical relevance. The present study was designed to find genetic variants associated with the outcome with an effect size of 0.2, which classify as a small to medium effect size (30). However, a great majority of genetic markers found in association studies have small effect sizes, and they explain only a small fraction of the genetic contribution to the diseases (31). Thus, it is possible that the genetic variants investigated in the present study are associated with LBP with radiculopathy, but the effect size is too small to be found with the present study sample size. However, it can be argued that if a common genetic variant does not have a measurable effect in a carefully phenotyped sample of 300 subjects it is unlikely that these genetic variants are clinically relevant as prognostic biomarkers.

Back pain was used as the primary outcome measure for LBP with radiculopathy to make the study more comparable with the GWAS and meta-analysis of chronic back pain. To ensure we captured all aspects of LBP with radiculopathy, secondary outcome measures specific for lumbar radiculopathy were also included. In contrast to the GWAS, where chronic back pain was analyzed as a binary trait, we used continuous outcome measures for back pain, leg pain and ODI. We do not believe that this explains the negative results, as using a continuous outcome measure would be expected to increase, rather than decrease, the power of the study. Most of the subjects had low pain intensity scores or low ODI, which could explain the weak or absent association between LBP with radiculopathy and the genetic variants. Sensitivity analyses were undertaken, with categorizing outcomes in recovery/non-recovery and in percentage change in outcome scores from baseline to 12 months, but as expected, the sample size in each genotype decreased to a level where we are underpowered to report the findings.

When using a genetic model to predict an outcome, one assumes that the outcome variable is a relatively stable measure. In the present study subjects were asked to rate their pain intensity for the past week, which may fluctuate from week to week, and thereby decrease the precision of the estimates. However, one can assume that such fluctuations are equally distributed across genotypes and thereby should not bias the results.

**Conclusion**

Our data did not support the hypothesis that LBP with radiculopathy 12 months after an acute episode of LBP with radiculopathy is associated with the selected genetic variants; *SOX5* rs34616559, *CCDC26/GSDMC* rs7833174 and *DCC rs4384683*. Therefore, the previously described association between chronic back pain and the selected genetic variants appear to be weak or absent in the present cohort. This suggests that the genetic variants *SOX5* rs34616559, *CCDC26/GSDMC* rs7833174 and

*DCC* rs4384683 are not useful as biomarkers for clinical decision making at an individual level for subjects admitted to a secondary health care institution.

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Table 1. Sample characteristics for genotyped subjects (n = 436) admitted to a secondary health-care institution due to an acute episode of LBP with radiculopathy. Comparison of subjects who responded to the follow-up questionnaire at 12 months with subjects who did not respond.

| Characteristics | Total sample (n = 436) | Included subjects (n = 338) | Subjects lost to follow up (n = 98) | p-value |
|---|---|---|---|---|
| Sex, males, n (%) | 251 (58.0) | 194 (57.2) | 57 (60.6) | 0.553 [a] |
| Age, years, mean (SD) | 42.7 (11.4) | 43.8 (11.4) | 38.6 (10.7) | <0.001 [b] |
| Education, > 12 years, n (%) | 322 (74.5) | 255 (75.4) | 67 (71.3) | 0.412 [a] |
| BMI, kg/m2, mean (SD) | 26.0 (4.3) | 26.1 (4.3) | 25.9 (4.5) | 0.788 [b] |
| Smoker, n (%) | 76 (17.7) | 51 (15.2) | 25 (26.6) | 0.010 [a] |
| Back pain, 0-10 NRS, median (IQR) | 6 (2.0 – 8.0) | 5.5 (2.0-8.0) | 6.0 (3.0-8.0) | 0.052 [c] |
| Leg pain, 0-10 NRS, median (IQR) | 8 (6.0 – 9.0) | 8.0 (6.0-9.0) | 8.0 (6.0-9.0) | 0.239 [c] |
| ODI, 0-100 %, mean (SD) | 49.3 (21.6) | 49.4 (22.0) | 49.1 (20.2) | 0.907 [b] |
| Pain duration, back/leg pain > 3 months, n (%) | 208 (49.1) | 156 (47.0) | 52 (56.5) | 0.106 [a] |
| Daily medication use, n (%) | 276 (63.2) | 216 (64.5) | 60 (63.8) | 0.908 [a] |
| Surgical treatment, n (%) | 218 (62.1) | 160 (62.0) | 58 (62.4) | 0.952 [a] |
| Back pain 12 months, 0-10 NRS, median (IQR) | 1 (0.0 – 3.0) | 1.0 (0.0 – 3.0) | - | - |
| Leg pain 12 months, 0-10 NRS, median (IQR) | 1 (0.0 – 3.0) | 1.0 (0.0 – 3.0) | - | - |
| ODI 12 months, 0-10 NRS, median (IQR) | 10 (2.0 – 22.0) | 10.0 (2.0 – 22.0) | - | - |
| Recovery, back/leg pain < 3 NRS 12 months, n (%) | 304 (61.1) | 304 (61.1) | - | - |
| *SOX5* rs34616559, MAF | 0.3 | 0.3 | 0.2 | 0.207 [a] |
| *CCDC26/GSDMC* rs7833174, MAF | 0.3 | 0.3 | 0.3 | 0.623 [a] |
| *DCC* rs4384683, MAF | 0.5 | 0.5 | 0.5 | 0.837 [a] |

Abberiavations: SD; standard deviation, BMI; body mass index, IQR; interquartile range (25% – 75%), ODI; Oswestry disability index, MAF; minor allele frequency.

[a] = Pearson Chi-Square

[b] = independent sample Student's t-test

[c] = Mann-Whitney U test

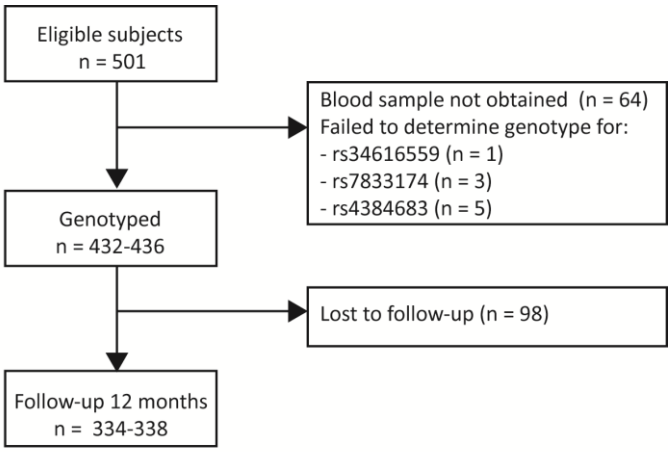


**Figure 1**. Study flowchart. A total of 334-338 (depending on the different genetic variants) was included in the final analysis.

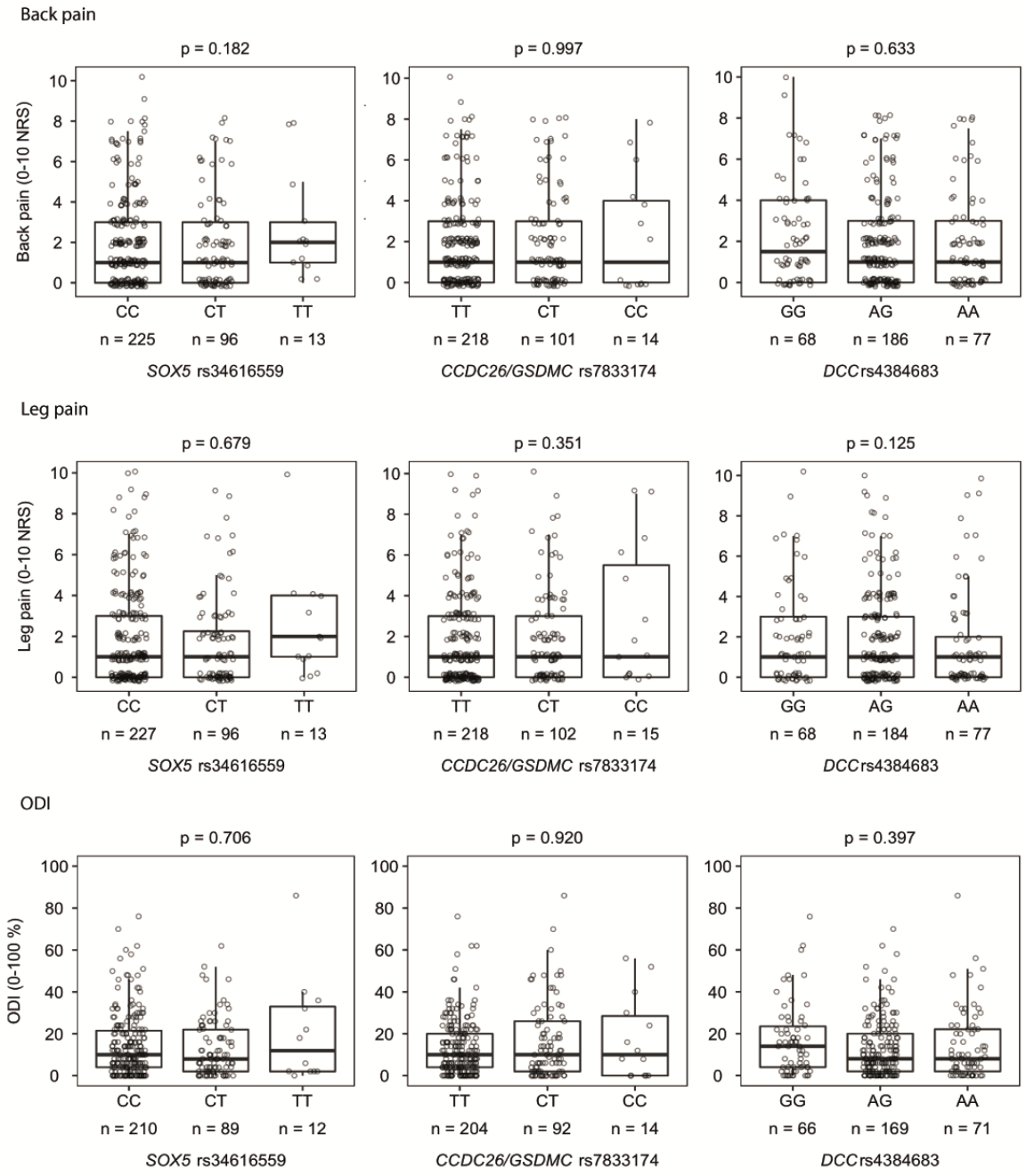


**Figure 2**. Associations between each genotype (3 allele groups) of the selected genetic variants (*SOX5* rs34616559, *CCDC26/GSDMC* rs7833174 and *DCC rs4384683*) and each outcome measure (back pain, leg pain and ODI) analyzed with Kruskal-Wallis H test. Missing data: Back pain (n = 3), leg pain (n = 1), ODI (n = 26). ODI; Oswestry disability index